

nature*July 29, 1976*

Making it in the Civil Service

It is now eight years since the Fulton Report on the British Civil Service advocated fairly comprehensive reforms in many aspects of the service, ranging from recruitment policies and staff training to job categorisation. Eight years is not a long period for an organisation with such inbuilt momentum as the Civil Service, but the Select Committee on Expenditure of the House of Commons deems it time to have a look at the way reform has been progressing. Last week was the turn of the Institution of Professional Civil Servants (IPCS), the body which represents professional, technical and scientific civil servants, to give its first evidence.

This past year hasn't been a particularly good one for the Civil Service. It has been the victim of a fairly regular stream of what the IPCS called "unfair attacks" and what others might call penetrating questioning of its role; the mutterings—and much more that hasn't surfaced—can be fairly clearly associated with the privileged position in which the civil servant finds him or herself at the moment. There has always, of course, been grumbling about job security and its dulling effect; the complaints have recently been about the way in which salaries and pension rights in the public sector seem to have become so much more favourable than those in the private sector, in which there is little job security.

It is, then, inevitable that what civil servants say about themselves and their situation is looked at in a rather hawk-eyed way; the IPCS at least didn't fall into the trap of telling the committee its members were underpaid. But they are, it was claimed, underprivileged—the words "second class" kept cropping up. In short, the IPCS believes there has not been a proper commitment by the Civil Service Department to implementing Fulton recommendations on removing vertical barriers between different classes or categories, notably the Administrative, Professional and Scientific categories. At the very top this has been done, at least formally, though whether it means much is questionable. What the IPCS would like to see is the removal of category labels much further down the system, in the hopes that this would allow policy-making and management prospects to open up at all levels for those who are at present confined by being 'professionals' or scientific'.

For all the IPCS's professed admiration for the administrators, this move is a thinly disguised assault on the

administrative citadel wherein, in the spirit of the genteel class warfare that is alleged to go on in the Civil Service, are housed the first-class characters who really have all the power and make all the decisions. Scientists, of course, run their own laboratories, so the call by the IPCS must be seen as an attempt to broaden the stage on which their members can operate.

There are problems. The first is that the IPCS, in making the point elsewhere in their submission that the distinction between generalists (that is, administrators) and specialists is artificial, acknowledges that administration contains a range of specialisms such as finance, accounts, contracts, personnel and computers and indeed that "public administration itself is a specialism". If we are all specialists now, the administrators will prove as defensive about opening up their ranks as would scientists about having administrators join them in some scientific venture.

The second problem is the sort of person who might be helped by such schemes. Outstanding professionals find their way to the top and go where they want to go without the need for much outside help. There is, however, a much greater number of what might be called the solid and reliable, and a real and as yet unsolved difficulty inherent in Civil Service use of professionals is how to prevent the solid and reliable from becoming disheartened, maybe as early as their mid-thirties, at the lack of prospects. But are there large numbers of jobs going begging in the administration categories or are there posts that ought to be created to allow these people to spread their wings? There are, after all, many solid and reliable administrators who are also likely to be in pursuit of such posts. The challenge then, is to the IPCS to do some extensive research and provide the Select Committee with some formidable evidence, down to chapter and verse, that there really are, as Fulton asserted, a significant number of jobs in the service, requiring both technical and managerial ability, which could be filled by technical people and which at present are not.

We have in the past advocated more radical approaches to the problem, particularly the golden handshake and enhanced mobility into and out of the service, but we believe that a detailed case is worth making for a different sort of solution. □



Tracking nuclear decisions (1)

JET: will it ever get started?

Chris Sherwell traces the tortuous course of a decision the EEC cannot take

WHEN, on the last day of last month, Mr Gaston Thorn relinquished the presidency of the EEC's Council of Ministers, he was frustrated and dissatisfied. The inertia paralysing the Community, he indicated, could be relieved by a move in the Council away from unanimous voting. It was the echo of a view expressed in many quarters many times before, though not perhaps by so senior or so obviously political a personage as the Luxembourg Prime Minister.

The widely-praised Heads of Government meeting two weeks later provided a much-needed boost for the Community. But its upper echelons are plainly troubled. Herr Guido Brunner, European Commissioner for Research, gave vent to his feelings when he visited London earlier last month. He too spoke of majority voting. But he had a specific matter on his mind—the Joint European Torus, JET. JET represents the next stage in the Community's effort to harness the power of nuclear fusion (see box, page 341). It also offers a remarkably fine illustration of what torments men like Gaston Thorn and Guido Brunner, not to mention scores of Community officials and countless people in the nine member states.

The immediate problem is that the Nine cannot agree to go ahead with the project without deciding on where it should be sited. The latest effort, at last week's meeting of the Council of Foreign Ministers, produced a piece of diplomatic mystification: the European Commission had asked for a decision of principle, before the summer recess, on the building of JET. This, it was thought, would facilitate a decision on the site when the Council of Research Ministers meets in October, and thus help prevent a split in the JET design team.

But the delays which have brought matters to their sorry pass were not ended. The Foreign Ministers watered down the Commission proposal, deciding merely to "adopt a favourable opinion on the rapid undertaking of the enterprise", which they thought should be put before the next Council of Research Ministers meeting for a decision. In short, the ball was put right back where it had been before, in the Research Ministers' court, and without any further movement over the site. This means that when a decision on where to site JET comes in October

—if it comes then—a full year will have been lost on a programme in which time is regarded as being of major importance.

What makes things worse, however, is that there must be some doubt attached to the prospect of an October decision for the precedents are truly inauspicious.

Euratom infusion

The Community's Fusion Programme, under the overall directorship of Signor Donato Palumbo of Italy, has its origins in the 1950s with the formation of Euratom and the EEC itself. Two five-year programmes were implemented between 1958 and 1967; work continued on a year-to-year basis between 1968 and 1970, when a third five-year programme was agreed. Once it was appreciated that, with the progress being made in fusion research, plans to build a large Tokamak—JET—could only be realised through an EEC-wide effort, a European design team of more than 50 members was brought together. The overall EEC fusion effort was co-ordinated by Euratom through a complex bureaucratic web of steering committees; JET is its centrepiece.

The laboratories at Culham, near Oxford, were offered as a location for the JET team. They form the United Kingdom Atomic Energy Authority's fusion research centre and, in the words of a Culham man, were offered "without prejudice to the final decision on the site for JET", which it was expected would be made by the end of 1975, when the original two-year contracts for the team's members expired.

All the advice the European Commission received in its wide consultations on the project acknowledged the risk attached to it, but the response was positive and members of the European Parliament gave their backing. So, with progress on the conceptual design of JET—a design which received additional backing at international meetings—the new overall plan for fusion research for the next five-year period (1976–1980) was put forward.

The total fusion effort for the period was put at 246 million units of account (mua). The estimated cost of the JET project was 135 mua—£80 million; the Community as a collective accepted responsibility for 80% of this (108 mua); member states would contribute the balance in proportion to GNP. Of the £80 million, some 21½% would go

on the JET device itself, 23½% on manpower, 17% on power supplies; the rest was for buildings (11½%), auxiliary systems (6½%), the operating budget (6½%), instrumentation (5½%) and contingencies.

As long as all these details were busily working their way up through committees towards the key arena of the Council of Research Ministers, and the customary problems involved in setting up a Council meeting persisted, a quick decision was not possible. With the putative deadline of 1976 looming closer, efforts were redoubled to secure a commitment on JET from the Council. But the Council of Finance Ministers, meeting first, would not decide on a financial allocation for a project on which a decision had yet to be taken. The Commission, facing the prospect of a sanctioned project not going ahead in the absence of a budget, obtained money to tide things over from the European Parliament under a special provision. It was a foretaste of the degree to which the Commission's ingenuity would be taxed. The Research Ministers finally met in December. And at that point the trouble really started.

See the sites

The meeting provided the first real indication that the Commission had completely underestimated the political problems involved. It emerged that Britain, with Culham, France, with Cadarache, West Germany, with Garching and Jülich, and Belgium, with Mol, all thought their own sites were suitable for JET. Italy, pushing for the Italian establishment of the Community's Joint Research Centre (JRC) at Ispra, refused to approve the whole 1976–1980 research programme in thermonuclear fusion and plasma physics unless a decision on JET's site was taken at the same time. No decision was taken, the programme was not approved, and the meeting broke up. Ahead of the next meeting, set for February, the Council's Atomic Questions Group exchanged views, and the Committee of Permanent Representatives examined its report, without taking anything further.

The difficulties were quickly compounded. Italy, having sought bilateral talks on the matter, failed to persuade Britain to support Ispra, and the British government started disclosing its own pro-Culham position publicly in the House of Lords. West Germany reasserted its willingness to take on 15% of the project's overall costs if Jülich or Garching was chosen as the site. Researchers at Culham reportedly began writing to Brussels urging an early decision.

That was not all. In late January, the Commission released the results of its

Confusion profusion: the arguments about the site

"If JET goes to Ispra, it is better that it does not get done at all." **Senior official, Culham fusion research centre.**

"If JET does not go to Ispra, it is not worth doing." **Senior official, European Commission.**

Culham and Ispra are probably the two main contending sites for the JET project. France's involvement in the Superphénix fast breeder is thought to make its bid for Cadarache more an attempt to score political points than a determined effort to win the project. Garching's candidacy is regarded with more severe respect and remains a powerful contender, but it has not planned to take JET in the way that Culham, with the design team, has done.

Only Italy defends Ispra with vigour; apart from Britain, though, none of the Nine openly backs Culham. The arguments concerning all the sites are finely balanced. The technical assessment of the Commission's site committee looked at the alternatives in terms of power supplies, infrastructure, safety and social facilities. On each criterion each site was fair to excellent: no site was best on all aspects, but all sites were suitable for the construction of JET.

It was chiefly on the power supply and social aspects that the Commission originally chose Ispra. It is fed directly from a conveniently located power supply; it already has an adequate infrastructure and social facilities, including an international school; it has staff already available for the project; and it has the equipment and expertise needed for the heavy engineering involved in handling large-scale plant and materials.

But the arguments in favour of Ispra now, if they didn't do so before, traverse broader territory than this. Perhaps the most potent actually comes from its supporters in the Commission itself, representing the collective Community interest. It is in the best long term Community interests, the argument goes, that the project be pursued as a Community effort. The project, particularly as it is largely Community financed, should therefore preferably be sited at a Community centre. And the premier establishment of the Community's Joint Research Centre (JRC)—indeed the only one capable of handling the project—is at Ispra, in northern Italy.

The argument goes further, into psychology. Ispra, it says, has been dogged by uncertainty in recent years,

and the latest programme of research is due to end this year. Although much of the multi-annual programme planned for 1977–80 will be conducted at Ispra, JET would insure its own future as the Community's leading research centre, which might in turn halt the fragmentation of research. Siting the project at Ispra would at the same time compensate Italy's loss by its exclusion from the UK–West Germany–Netherlands Gas Centrifuge Treaty for Uranium Enrichment.

The case against Ispra has come mainly from Britain, a point that has not gone unnoticed amongst senior Community officials. It turns most importantly on the project's chances of success. The chief scientist at the Energy Department, Dr Walter Marshall, not known as a fusion enthusiast, puts it this way: there is "an appreciable chance" that the project will fail, not because it is badly conceived but because it is so ambitious; it should not therefore go to any site which "lacks experience and know-how in solving the plasma physics problems that are bound to arise." Ispra's fusion experience (often described in Britain as "modest") is not great.

Marshall does think that the project should go ahead. But he judges that other sites, including Culham, satisfy his criterion in a way that Ispra does not. Moreover, he is "very much frightened" that if the counter-arguments about symbols of European collaboration win the day, then the project really will be a failure. If he could be persuaded of the scientific capability of a site, he has said, he would recommend it, but this he says has not been done; there had only been a "review of the characteristics" of the sites.

The implication is that a site (like Ispra) ought not to be chosen for an additional reason—to solve an administrative problem. Others allege in addition that Ispra's resources, and its capacity to get them, are not great. It has even been said that scientists would be reluctant to work at Ispra, especially as it has not managed a large project before. Past labour problems add to its reported reputation for mismanagement—a reputation also pinned on the Commission, whose allegedly closed mind over the matter has proved a sore point in Britain. That Ispra could prove to be the most expensive site, which is also suggested, is a point that Guido Brunner, European Research Commissioner, disputes. He

says the differences in cost between the various sites is no more than 10%.

The arguments in favour of Culham are partly the arguments against Ispra reversed. Culham, it is said, is already a centre of excellence in fusion, commanding large experience and resources, possessing a good "track record" and making progress that is more than comparable with the USA and USSR. Furthermore, it not only fits the bill on such essential criteria as power supplies, it also has the land ready to take JET and, most importantly, a team already settled there to continue the work it has successfully begun. Such continuity, it is argued, ensures the maximum chances of success. Culham, says Alex Eadie, junior minister at the Energy Department, is the best site on technical, scientific and operational grounds. But another reason offered in support of Culham is more nakedly political: namely, that there is as yet no high technology Community project in Britain. This gives it some sort of advantage over Garching, for example, since West Germany has the Patent Office and the European Molecular Biology Organisation.

Arguments against Culham throw doubts on its back-up facilities, on the lack of a school and so on. Beyond these, though, it is said that expertise in fusion is not really relevant for much of the project's duration, since most of the time would be spent in JET's construction; moreover, any problems that arose would probably be too big for any one laboratory to solve alone. More subtle arguments point to the recent experience with the Dragon high temperature reactor project at Winfrith, Dorset, during which Britain attracted from many European countries criticism of its foot-dragging, if not obstructionist, tactics over the project's fate.

The site stand-off preventing JET's take-off is thus a complicated affair. It has tried the patience of the contending host countries, whose Community spirit is already under scrutiny. It has aggravated the frustrations of the smaller member states who these days have enough reason to feel left out in the cold. And it has threatened the role of the Commission, whose ambiguous position has rendered its influence and authority more tenuous than it might otherwise have been. The Community's worst enemies couldn't ask for more. □

independent siting committee. It had not previously pushed any one site; now it came down in favour of Ispra. Moreover, it delivered its view as a communication instead of as a "proposed decision" for the Council, as was customary; this it defended by saying that only the whole project, including the matter of the site, and not the site alone, involved a decision. The boundaries of the Commission's competence to decide on the matter thus became an issue as well.

The February 24 meeting of the Research Council began by avoiding an immediate break-up over the site. British, French and West German pressure wrought a reduction in the total fusion budget from 246 mua to 232 mua, thereby leaving less for the fusion research not directly related to JET. This, however, entailed the abandonment by Italy of the position under which she had linked a decision on the site with approval of other fusion research programmes; but she still insisted, successfully, that the meeting sanction just one year's expenditure—20.8 mua. But that at least meant the programme could go ahead.

When it came to discussions of the site, however, none of the contending countries withdrew their applications to host the project. After hours of debate, the Ministers failed even to agree on a method for deciding where JET should go. They rejected a proposal that the Commission, as the competent authority, should decide, and also the idea of an exhaustive ballot among themselves. Eventually, applying an old adage ("When in doubt, never commit yourself, committee yourself"), they agreed to call for a new report on the alternative sites. This was to come from a special committee established by the meeting, the Consultative Committee for the Fusion Programme, consisting of a representative from each of the Nine, a Commission representative and representatives from third countries involved in the fusion programme. It would report on comparative technical merits in May, in time for the next Council meeting, set for mid-June.

Some states openly regarded this as a waste of time; Italy hoped that the question of the site would not be reopened. For the Commission, Brunner still claimed the matter was within its competence: he couldn't see what new light would be thrown on the problem by more technical assessments; only something very dramatic could change the Commission's mind. For all its immobility though, the Commission did not apparently intend to try and impose its choice.

By now the threat of rising costs was starting to make speed vital; so too was the ground being lost against the USA and USSR; and the team's declining

morale, with the increasing prospect of its break-up in spite of a renegotiation of contracts for a six-month extension. But the rest of the fusion programme was assured for a year; and the new committee had also been accorded a general role in respect of the whole programme, which would help its development. All the same, it looked as though the JET problem was so intractable that it would need resolution by the Council of Foreign Ministers or by the nine Heads of Government—a suggestion which the Dutch had by now put forward.

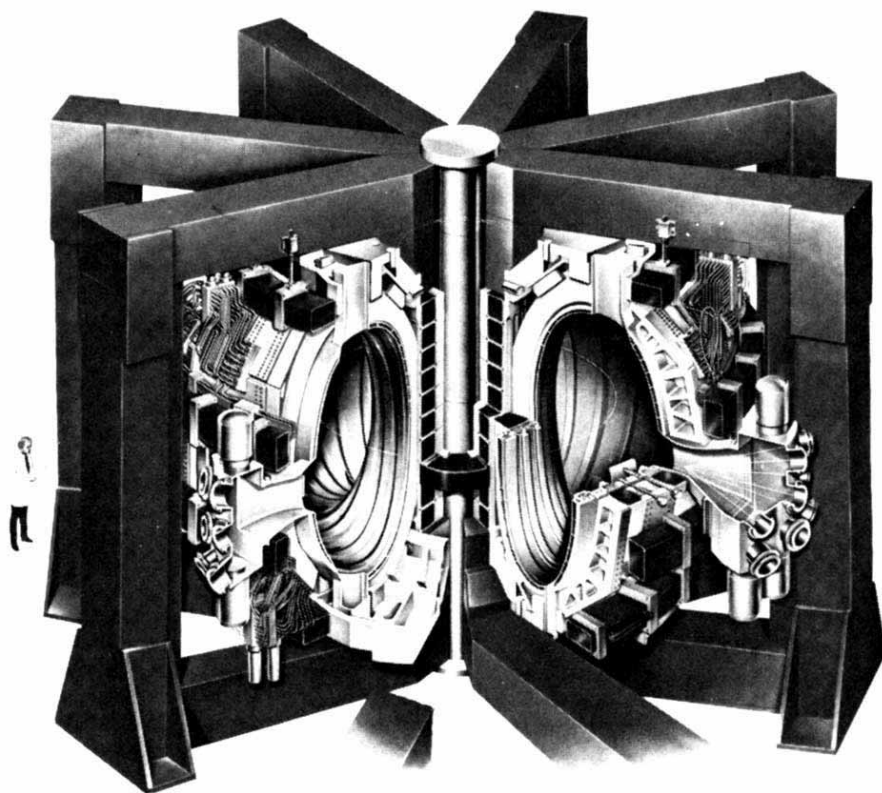
Fight goes on

With the fighting now well developed, two sites were withdrawn—Mol in Belgium and Jülich in West Germany. Reports of job offers from the USA for members of the design team grew, and a few actually left the project. Britain made a climb-down more difficult when she put her view favouring Culham to the House of Commons in March. The "common front" of trade unions at Ispra called a press conference later that month to urge a decision in favour of Ispra and of a Europe independent in energy and science.

The Consultative Committee, at its first meeting in early April, received an independent report on JET's design details from the UK-based Risley

group. This confirmed the team's plans, and the Committee recommended that a decision to build was needed forthwith. It recommended too the immediate creation of a management committee for JET together with a strengthened fusion directorate at the Commission. But the Committee also heard estimates that certain costs had drifted upwards, making overall costs some 23 mua greater than the original 135 mua. Another meeting was set for mid-May to discuss a final report following consultations with the JET team over the Risley report. Incredibly, the Committee did not discuss the matter of the site.

Neither was it discussed at the Council of Foreign Ministers meeting in early May, even though Luxembourg, backed by Belgium and Holland, was trying to put it on the agenda. In fact the agenda was already too full. The lack of movement encouraged some European parliamentarians to speak out volubly, questioning whether European collaboration was possible, and whether latest estimates of Europe's future energy requirements didn't tilt the balance one way or the other. In mid-May Brunner signed the draft agreement on Sweden's participation in the fusion programme; negotiations with Switzerland, something which with the Swedish precedent was not expected to take long, were almost



EEC picture

JET: an artist's impression

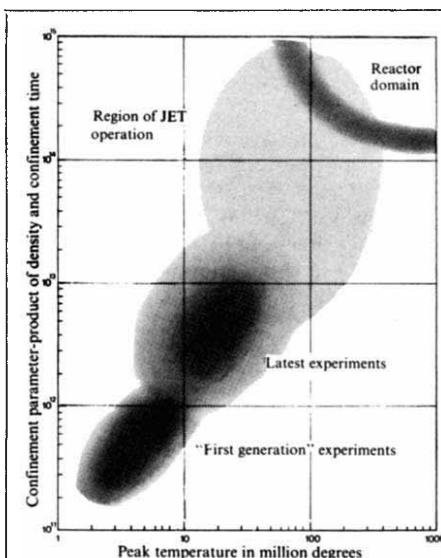
ready to commence and soon received the go-ahead. The pace was quickening again.

The Italian general election, set for June 20, just two days after the crucial Council of Research Ministers meeting, now intervened. With the prospect of an upset result producing an Italian government which might disagree with any decision taken by the Council, it began to seem pointless having the meeting at all. At Italy's request it was decided that the meeting would not take place. The Dutch proposed a meeting for mid-July; this was agreed in principle. As late as the first week of June, Brunner was expressing the hope that a decision on the site would be taken at such a meeting. Days later it was clear that no meeting was likely before the summer recess. No early decision on the site would therefore be possible unless the idea of a determination by the nine Foreign Ministers was resuscitated.

The Commission had itself come round to this view. It had also been busy on the central problem of the site: by the end of May it had finished preparing additional information for the Council of Research Ministers in order to make the choice easier. This included, first of all, the news that consultations concerning the higher cost estimates had produced agreement that the extra amounts involved were in fact negligible, at about 5 mua. The Commission also contended that insufficient emphasis had been attached previously to the matter of the power supply: if JET was to be fed directly, Ispra, fed by a line from a thermal and a hydroelectric station, possessed another advantage over other sites. Finally, the Commission stressed the costs of delay. Increasingly frustrated, it therefore argued that no attempt to justify a further delay could be based on arguments of a scientific, technical, financial or administrative nature.

With no prospect of a meeting of the Council of Research Ministers before mid-October, and the need for decisions growing more desperate, it was now time to change tack. At the end of June, therefore, and at the request of the Dutch (who were about to take over the Council presidencies), the nine Foreign Ministers had a brief exchange on the siting of JET. The Commission was now looking for a decision of principle on the building of JET before the recess in order at least to supply more adhesive to the JET team than the second six-month extension to contracts just put together.

The matter was put on the agenda of the Heads of Government meeting on July 12. The boost they were busy giving to the Community did not extend as far as JET, however, and it was not discussed. Assurances were



Fusion research: where it's at

FUSION research, which seeks to harness the energy released when nuclei of light elements are brought together to form heavier ones, aims ultimately to design a practical fusion reactor that produces electricity economically. The aim is thus to approach more closely the conditions at which the energy released from the deuterium-tritium reaction (the fusion reaction selected as the most promising of several possibilities) is greater than that expended both in heating the plasma in which the reaction occurs, and in losses.

The immediate goals are therefore to find ways of heating the plasma to the necessary temperature, and to confine that plasma for the necessary time. Apart from the passage of electric current, methods of heating currently being investigated include the use of intense laser beams and the use of beams of high-energy neutralised particles originally produced from an ion source. The method of confinement in a doughnut (torus) shape uses a helical magnetic field. This is made up of two components, a toroidal field produced by large external coils, and a poloidal field which, in the case of tokamaks like jet, is produced by a toroidal current in the plasma.

Tokamaks, however, represent only one of three classes of toroidal device now being examined for their confinement capabilities. These are in fact distinguished by the way the poloidal field is produced:

(a) **Tokamaks, and Reversed-field Pinches** (high beta systems). In tokamaks the toroidal field is greater than the poloidal field; in reversed-field pinches the two are about the same and the toroidal field is reversed in the outer

regions of the plasma. In both, the plasma current which heats the plasma also creates the poloidal field.

(b) **Stellarators** (low beta systems). In this class of device, currents in helical conductors wrapped round the torus and outside the plasma produce the field which, with the toroidal field, confines the plasma.

(c) **Toroidal multipole**. Here the poloidal field is produced by a steady current flowing in a levitated superconducting ring located in the centre of the vacuum chamber with the plasma.

Magnetic confinement produces a plasma of modest density, so the confinement time necessary to boost the "confinement parameter" (density x confinement time) is longer compared to a plasma of very high density.

Obtaining a high-density plasma is the aim of inertial confinement, in which the necessary time of confinement is actually shorter than the period in which the particles will escape from the reacting zone. This confinement is achieved by irradiation with high-power laser beams. Extremely intense, short laser pulses bombard a solid deuterium-tritium fuel pellet, causing it to turn into a plasma so hot and so compressed that fusion reactions yield a surplus of energy before the pellet decays as a result of expansion. The method does not simply offer the possibility of avoiding the difficulties of using magnetic fields for confinement; it also offers the theoretical possibility of using a series of laser pulses to cause a succession of these explosions.

The selling of fusion power has turned largely on its ostensible advantages in respect of the fuel it uses and its safety. But the supply of lithium (used to produce tritium) is at least not certain, and neutron production in the reactor has the potential to make the reactor materials highly radioactive. There are other problems relating to possible lithium fires and tritium leakage. And since fusion is so patently a prospect for the distant future, guesses about its economics remain exactly that—all of which makes any choice between fusion and fast-breeder fission, if such a choice exists, correspondingly more difficult to make.

JET is effectively a "third generation" tokamak device. Its equivalent in the USSR is known as T-20, in the USA as TFTR, and in Japan as JT-60. "Second generation" tokamaks include DITE at Culham, T-10 in the USSR and PLT in the USA.

For the post-Jet period, the following sort of sequence is imagined:

1980-1985 Tritium burning experiment
1985-1990 Experimental reactor
1990-2000 Prototype
2000-2010 Demonstration

Other common facilities envisaged over this period include a material testing facility and a superconducting magnet assembly.

JET lag importance

The persistent procrastination, far from indicating that the EEC perceives the JET decision as having comparatively minor consequence, is precisely a reflection of the project's importance. JET is a major Community research project, perhaps the first of its type,

given that last week's Foreign Ministers meeting would deal with the matter in some detail though it was not on the official agenda. Nothing approaching progress which would satisfy the Commission resulted. The recess is now imminent. October is two more months away.

perhaps the first of many. Money is to be spent, and there are contracts to be had. That means large and powerful interests are involved, not just on both sides of various sectors of manufacturing industry, but within state administrations and institutions and within the EEC's own administrative structures.

With so much at stake, the decision on the site had to become the prime focus of interest. To win JET was (and is) to win fusion in Europe. There are some, notably the Italians, who have claimed that JET, being just one part of an even larger overall programme, would not by its location determine where the European centre of excellence would lie. The argument is that the other projects to come would be sited elsewhere. But no one seems to believe it. Whoever wins JET, most are saying, acquires the huge investment that goes with it, the location for future reactors and the investment that goes with them too; whatever the spreading of the contracts, and no matter what clauses are inserted as safeguards into the agreement, the host country is said to have the advantage. That country's cap acquires the fusion feather—and the reputation, the prestige and the national pride. It seems rather a lot to hang on a unanimous decision of a group of Research Ministers.

The Commission, in the form of Signor Palumbo, finds all this a trifle exaggerated. He claims that the press has "transformed a technical and limited problem into one of prestige", and points out that there is another aspect of the problem beyond the interest of a country in having JET. This is the interest in having JET at the best possible site. Suppose, he says, a super-intelligent, objective man

could identify both the best and the worst sites among those competing for JET. The difference between the value of the best site and the value of the worst site, he argues, is hugely outweighed by the damage done by a one-year delay to the JET project and to the common fusion programme. Indeed, all sites could host JET. The choice of the site, while blocking everything, is thus irrelevant, he claims, and he goes on to repeat the growing view—that if the Commission's choice is not approved, the Council of Ministers must give up its jealously guarded unanimity rule to come to a definite choice.

He certainly seems to be right about the urgency. Existing programmes for medium scale devices at Jülich, Cadarache, Garching and Culham all depend technically and financially on a decision on the site for JET. Delay is discouraging the JET staff even further, making it difficult to maintain the team and impossible to expand it. It has been possible to farm out some small study contracts relating to the project, but money available for this is almost totally spent, and a Council decision is required for more. Plans for buildings cannot be finalised, of course, buildings cannot be built, and orders cannot be placed for equipment that might be unsuitable or superfluous if another site is chosen. As the Commission has itself put it, any decision concerning further work without the site being known would be unrealistic, perhaps dangerous and almost certainly expensive.

Just how expensive is perhaps not appreciated. The delay, apart from jeopardising the project itself and the whole fusion programme, may now be jeopardising the spirit of cooperation

that exists in Europe. It may also be damaging the credibility of the Community's decision-making procedures among other international organisations and in other non-EEC countries. Most importantly, it may soon start affecting Europe's own public, whose tacit support for the expenditure of so much money is less likely if the persistent delays (and the corresponding progress of the Americans and Russians) make the project seem pointless.

Optimists point out that a decision in October would mean that a delay of only a year had been incurred. No decision in October might mean leaving the decision as late as the next Heads of Government meeting in The Hague in December. But the viability of the whole project may have been brought into question by that time: as Herr Schuster, Director General at DG XII in Brussels, puts it, if there is no decision by then, the project is dead in an EEC framework. Yet it is Schuster who freely acknowledges that the German elections later this year might intervene, Italian-style.

An alternative has already been mooted: that JET becomes a British-French-German operation. But a British minister has already said Britain is committed to European collaboration. And the Commission heaps derision on the idea. Alternative combinations, it is said, are "futurology": the three countries can't agree among themselves, and JET is anyway "owned 100%" by the Commission. But political facts could conceivably change things. The Community is being brought into disrepute. Fusion itself is being brought into disrepute. Unless something is done, a Community fusion project might therefore be a forlorn hope. □

Tracking nuclear decisions (2)

FBR: will it ever be stopped?

Allan Piper looks at the arguments concerning the fast breeder reactor

THE UK Energy Secretary, Anthony Wedgwood Benn, decides this autumn whether Britain will build a commercial scale fast breeder reactor. It is a measure of the task he faces that, from one viewpoint, the FBR is an elegant and timely solution to immediately foreseeable energy problems, while from another it looks like potentially the most disastrous technological development imaginable. The dichotomy is simply explained: the reactor's most attractive advantage—an ability to create its own fuel—is precisely its major disadvantage. For the fuel so created is plutonium, the stuff

of nuclear weaponry.

Mr Benn is fully aware of the enormity of his decision's implications, whatever he chooses. Not much is known of how he will reach it, beyond the fact that a certain amount of public debate is involved. Some people believe the decision is being taken too quickly and ought to be delayed, saying the FBR is not yet needed; others contend that an early decision is vital for the industry and the country. The arguments are complicated, but what Mr Benn has to decide is, first, whether fast reactor technology is an essential element of the future energy scene;

second, whether it can be developed to an acceptable level of safety; and, finally, whether it is socially desirable. He must make his decision against a backdrop of clear signs that the FBR will be developed elsewhere if not in Britain, and delay could spoil the chances of an early foothold in a potentially lucrative worldwide market.

The case for the FBR rests squarely on its fuel breeding capacity (see box, page 344). Unlike existing commercial thermal reactors, which use less than 1% of the energy available in uranium, the FBR extracts more than 60%. At the same time it contributes towards future energy supplies by "breeding" plutonium. These simple advantages mark it out as the potential saviour of an energy hungry world.

Consideration of Britain's nuclear fuel prospect highlights the possible benefits of the FBR. Since the middle 1950s the UK thermal reactor pro-

gramme has produced 20,000 tons of depleted uranium. Ordinarily of little value, this stockpile, together with the 13 tons or so of plutonium that have also accumulated, could be used in fast breeders to provide as much energy as the nation's total known reserves of coal. For a country with a sizeable (5 GW) and expanding nuclear programme, this represents an indigenous energy source that could become ever more crucial as the domestic requirements of the uranium exporting nations themselves increase. British imports from Canada could be squeezed, as could possible future supplies from Australia. A large political question mark hangs over Namibian uranium, Britain's only other source of reactor fuel.

Worldwide nuclear expansion will also introduce economic pressures. Present assessments of world uranium reserves are as elastic as the market prices assumed in calculations, but at the existing price of around \$15 a pound an estimated 3.4 million tons of high grade ore are available, though only half of that is so far proven. Whether this can last beyond 1990, even with today's worldwide nuclear capacity, is already a matter of debate.

There is less doubt, however, that by the turn of the century the continued use of thermal reactors alone could force the exploitation of lower grade ores. The consequent increase in prices, which might rise as high as \$100-\$200 a pound if forecasts from the US Energy Research and Development Administration prove correct, is likely to be matched only by the increased amount of environmental despoliation accompanying the extraction of ores with energy contents similar to that of coal. (Currently used ores provide around 25,000 times as much energy as coal in thermal reactors, or up to about 1.5 million times as much in fast breeders.)

What bearing this has on the case for a British FBR programme is rather a matter of viewpoint. On the one hand, energy demand forecasts from within the nuclear industry and the Department of Energy (DEN) identify the FBR as an indispensable cornerstone of the British energy scene until fusion power arrives. On the other, it is easy to query the accuracy of forecasts. The degree of uncertainty involved was underlined during the recent national energy conference when British Gas leaders refuted predictions of a UK energy gap before the end of the century.

Independent research units similarly believe that electrical energy demands may already be near to peaking, while pressure groups claim that demand can be regulated by conservation measures, ensuring that sufficient nuclear capacity



Prototype Fast Reactor with the Dounreay Fast Reactor in the background

is achieved economically using more efficient thermal reactors systems such as CANDU (see box). There are also calls for more research and development (R&D) effort on high temperature reactors, which open up the possibilities of the thorium cycle. But the UK Atomic Energy Authority (UKAEA) is less enthusiastic, wary of the huge initial uranium investments needed to launch such a programme.

UKAEA forecasts predict that by the turn of the century almost 75% of the national energy demand must be met by nuclear power. Of the 198 GW needed to do this job, almost 33 GW, nearly seven times the current total installed nuclear capacity, will come from fast breeders. Whether this target is treated entirely seriously by the DEN remains doubtful, particularly as the Central Electricity Generating Board (CEGB) could by then handle a maximum of only 20 GW from FBRs. Moreover, Walter Marshall, Chief Scientist at the DEN, has several times publicly voiced the conviction that the number of commercial fast breeders operating by the year 2000 is unlikely to exceed two or three.

Nonetheless, this only reinforces the UKAEA's case for early clearance for their planned 1,300 MW demonstrated FBR, CFR-1. It has built up a good head of steam for the off. Its R&D effort on FBR technology extends back over 25 years, with operational experi-

ence dating from the start-up of the 14 MW experimental FBR at Dounreay in Scotland (DFR) in 1959. The programme advanced a further major step two years ago with the introduction at low power of a 250 MW prototype (PFR), also at Dounreay.

Estimates of the total cost involved in coming thus far vary between £200 and £400 millions, but the UKAEA's present level of financial commitment speaks for itself. Though cuts of up to £4 millions are expected as part of current UK expenditure savings, R&D spending on FBR technology over the past three years has stood at around an annual £40 millions, almost a third of the total nuclear R&D cake, and by far the largest single slice. Moreover, about half of the overall nuclear R&D workforce is involved with the FBR.

The UKAEA therefore understandably feels that the next logical step is the design and construction of CFR-1, expected to take about 10 years. Confidence generated by almost two decades of reasonable progress with DFR, which has pumped more than 500 kWh into the national grid, extends beyond the reactor itself, into every area of the related fuel cycle.

At least one group does not share the UKAEA's confidence. Friends of the Earth (FOE) have vocally attacked the international record of fast reactor development with single minded conviction. During a recent House of

Commons Select Committee meeting FOE listed a series of developmental setbacks that provokes sober reflection. The Enrico-Fermi FBR in Detroit, Michigan, for example, was constantly beset by potentially dangerous disruptions during its sporadic, nine-year operational life, while more recently the Soviet BN-350 FBR on the Caspian coast has suffered several serious leaks in the steam generating plant. Welding has, indeed, proved a persistent problem, and has delayed the full-power operation of the Dounreay PFR. The

molten sodium used in the cooling circuits is highly corrosive. Void formation—the occurrence of gas bubbles in the coolant—is another major problem common to all FBRs and has still to be overcome.

Remaining hitches in reactor technology can, it is argued, be straightened out during the 20-year run-up to a commercial FBR network. But doubts linger over the associated fuel cycle. They arise not only at the technological level, where experience already gained by British Nuclear Fuels Ltd has

been encouraging, if somewhat controversial, but also at environmental and political levels.

In a submission last year to the Royal Commission on Environmental Pollution (RCEP), which is due to publish a report on radiological hazards before Mr Benn's autumn decision, the UKAEA covered the environmental issues. Its arguments that the hazards of long-lived, highly active wastes can be satisfactorily controlled are likely to tip the scales in favour of the FBR. Mr Benn has indicated that the RCEP report will influence his decision, and it is now clear that the Commission itself, for some time reportedly divided over nuclear issues, will back CFR-1. In a recent reference to commercial FBR development Sir Brian Flowers, Chairman of the RCEP, said that while the implications and alternatives had not been explored with the level of resources and dedication devoted to the nuclear programme itself, he would not oppose the building of CFR-1 alone.

But the social and political implications of FBR reprocessing most worry those who believe that one step forward to CFR-1 will carry too much momentum for subsequent withdrawal. Though FBRs produce plutonium at a slower net rate than thermal reactors their proliferation would introduce the need for widespread plutonium transport. On UKAEA reckoning there could be 250 tons at various stages of the fuel cycle in Britain by the year 2000.

FBR opponents fear that the security measures required would be not far short of Draconian. A Bill to introduce the arming of nuclear security police has already been passed by the UK Parliament, and a US Government official this month called for an armed international security force. Additional doubts have been expressed about the sort of political arrangements needed to ensure worldwide adherence to safeguards such as those already implemented on a more limited scale by the International Atomic Energy Agency.

In France, the political and technological fears have led to civil disturbances involving qualified and well-informed opponents of the 1,200 MW Superphénix project now beginning on the Rhône. But in Britain there is little public awareness of the issues. Sir Brian Flowers recently charged that the UK Government apparently preferred to keep things in the dark. Similar concern has also led the Liberal Peer, Lord Avebury, to call for a Green Paper once the RCEP report is available, and to hint that a national referendum would not be inappropriate. Mr Benn has said only that a questionnaire will be produced for limited, and so far unspecified, circulation.

Though it is clear that the case for

How the FBR is different

NUCLEAR power installations harness the heat energy released during controlled nuclear fission, and convert it into usable electricity. Fast breeder reactors (FBRs) differ from existing commercial reactors (known as "thermal" reactors) because, along with energy, they produce more fissile material than they consume—a self-sustaining bonus which could considerably stretch world uranium resources.

Like fast breeders, thermal reactors produce some plutonium but only in minute amounts. This is because more than 97% of the uranium used in thermal reactors fuel is non-fissile uranium-238, which is partially converted into plutonium while fission of the small fraction of uranium-235 proceeds. But plutonium is itself fissile, and most of it rapidly breaks down again, adding to the overall energy output of the system. (The Canadian CANDU reactor is designed to take full benefit of this phenomenon, optimising fuel efficiency.) Thus only about 1% of spent fuel from thermal reactors remains as plutonium.

On the other hand, the FBR can breed plutonium, using fast neutrons rather than the slow neutrons used in thermal reactors. Although fast neutrons are less efficient in inducing fission, the chain reaction they set up produces the greater number of additional neutrons needed to convert uranium-238. Consequently, the moderators used to decelerate fast neutrons in thermal reactors are absent from fast breeders.

The problem of decreased fission efficiency is overcome by pushing the neutron density in FBR fuel elements above the level achieved in thermal reactors. This means that the core assembly must be extremely compact, while the concentration of fissile material must be boosted way above the maximum enrichment factor of 3% needed in thermal fuel.

The 14 MW experimental FBR at Dounreay (DFR), for instance, uses 75% enriched metallic uranium in a core of little more than 100 litres. At Dounreay, as in all FBRs, the uranium-238 needed for breeding is located in a "blanket" around the core, where the neutron density is no longer sufficient to break down the fissile plutonium formed.

In assessing the doubling time of an FBR—the period required for the reactor to reproduce the amount of fissile material originally in the reactor core—the entire fuel cycle is considered. At any one instant, for example, fissile material may be within the reactor itself, undergoing cooling, reprocessing or re-fabrication, or in transport.

It is thus possible to control the doubling time by retiming any of the operations involved in the cycle. Current UKAEA plans envisage a doubling time of about 28 years for Britain's first commercial FBRs early next century, a figure that neatly matches the expected expansion rate of the reactors themselves.

With the high neutron density in an FBR core, heat is generated more efficiently than in thermal reactors. Thus, while most thermal reactors operate at temperatures well below 300 °C, the 250 MW prototype FBR at Dounreay (PFR) has a design outlet temperature of almost 600 °C. Fast breeder fuel elements must be designed to withstand the extra heat.

It soon became apparent that the metallic uranium core used in the DFR would melt at operating temperatures above 350 °C. As a result, the PFR is fuelled by sintered pellets of uranium oxide and plutonium oxide. Uranium-235 is not an essential component, however, and fast breeders of the future could be fuelled with fissile plutonium-239 alone, though uranium-238 breeding material would, of course, have to be present. It is in by-passing the need for the scarce uranium-235 isotope that the FBR can stretch available uranium resources.

The fast breeders so far developed in Britain, France, Germany, Japan, the USA and the USSR are all designed to use liquid metal coolant circuits to carry the heat away from the core. They are therefore known as liquid metal fast breeder reactors. By using sodium, which is normally molten between 98 °C and 880 °C, the unnecessary hazards associated with pressurised operation procedures can be avoided.

To minimise the risk of radioactive contamination, sodium circulated around the core in the primary reactor chamber passes its collected heat onto a secondary, external sodium circuit shielded from radiation. This circuit in turn carries heat to the steam generators.

FBR development has not run altogether smoothly. After 17 years operational experience with the DFR and the PFR, the UK nuclear industry is still struggling with problems in the steam generating plant of the PFR. The CEBG, however, which is prepared to have 20 GW of FBR capacity plugged into the national grid by the year 2000, has also declared a watchful interest in the development of helium-cooled fast breeders, which could provide an even better alternative to the liquid metal FBRs.

the FBR is far from open and shut, Mr Benn's view of international developments may persuade him to nod CFR-1 through. France, Germany, and the USA are all aiming for commercial FBR capacity by the 1990s, with Japan and Russia not far behind. UKAEA collaboration agreements already exist in one form or another with all of these countries, while the CEBG, currently chasing a minor share of the Superphénix project, also holds an interest

in the German SNR-30 FBR prototype.

UKAEA feeling is that delays with CFR-1 will not help strengthen collaboration, perhaps damaging Britain's relative position in a commercial development race that will be run anyway. One man who disagrees is John Surrey, who last month resigned as nuclear adviser to the Select Committee on Science and Technology. He has argued publicly that Britain can learn

much from the sidelines, and that restraint at this stage will allow the channelling of larger funds into other areas of energy R&D, including conservation. The UKAEA must hope that Mr Benn is unimpressed by that reasoning: it has already placed a contract with the Nuclear Power Company for design and engineering work "related" to CFR-1. On the question of a possible site, there has been official silence. □

Tracking nuclear decisions (3)

Exports: time for a stand?

Colin Norman in Washington examines the NRC's problems concerning the export of uranium to India

EARLY next month, the Nuclear Regulatory Commission (NRC) will decide whether to allow 12,261 kg of slightly enriched uranium to be exported to India. Its decision, the toughest it has yet faced, will have major foreign policy implications, for it will represent a crucial milestone in the United States' efforts to prevent the proliferation of nuclear weapons. In fact, it is the kind of foreign policy decision that is usually made by the President.

NRC is thrust into the middle of the issue because it alone has the authority to grant or deny an application for a licence to export nuclear material from the United States. Its decisions can only be overturned by an Act of Congress. In this instance, it has under consideration an application to export fuel for the giant Tarapur Atomic Reactor Station near Bombay, an American-built reactor which has been operating since the early 1960s with fuel supplied by the United States.

Understandably, NRC is treating the matter with considerable caution. Last week, it held a public hearing to receive testimony on the licence application—the first public hearing ever called to discuss a nuclear export licence—and it was given a wealth of conflicting advice. On the one hand, the State Department and the Commission's own staff recommended that the application be approved, while on the other, a powerful coalition of arms control experts, Congressmen, environmentalists and nuclear critics argued that the licence should be denied.

Underlying the debate, of course, is the fact that on May 18, 1974, Indian scientists exploded a nuclear device constructed from plutonium produced in a Canadian-supplied reactor. It was the first time that any nation had used imported technology to join the nuclear club, and as a result Canada earlier this year decided to bar any further

nuclear assistance to India. Opponents of the request to ship enriched uranium for the Tarapur reactor are urging that the United States should follow Canada's example.

Representative Clarence D. Long of Maryland for example, told NRC that "our response to India is the first test of whether the United States has a real policy of stemming the spread of nuclear weapons". Similar sentiments were also expressed by Dr Herbert Scoville, former deputy Director of the Central Intelligence Agency, and Adrian Fisher, former chief negotiator for the Nuclear Non-proliferation Treaty (NPT). Fisher argued, for example, that "the continued supply by the US of nuclear fuel for the Indian atomic program can only be looked on by other nations as tacit approval by the United States of the Indian nuclear explosive program".

The United States has been supplying enriched uranium for the Tarapur reactor for more than a decade under a unique agreement. In short, the agreement specifies that the reactor can only be operated with fuel supplied by the United States, and that such fuel cannot be used in any other facility in India. The United States also has an option to buy back spent fuel discharged from the reactor—nearly 200,000 tons have so far been accumulated—and no reprocessing of that fuel can take place in India without US permission. Moreover, operation of the Tarapur reactor is subject to monitoring by the International Atomic Energy Agency (IAEA).

But India has an extensive nuclear programme in addition to the Tarapur reactor which is not covered by the agreement and which is not subject to international safeguards. The explosive device detonated in 1974, for example, was made from plutonium produced in a Canadian-supplied research reactor and separated in a small reprocessing

plant built by the Indians themselves. And a new factor has recently been introduced because India has recently completed construction of a large reprocessing facility adjacent to the Tarapur reactor. According to State Department spokesmen at last week's NRC hearing, the facility is now undergoing tests and it will soon have the capacity to reprocess much of the spent fuel from India's entire nuclear programme. The construction of the plant has given India at least the capacity of building large numbers of explosive devices.

Opponents of the proposal to ship more fuel for Tarapur, led by the Natural Resources Defense Council, the Union of Concerned Scientists and the Sierra Club, argued at last week's hearings that, at the very least, NRC should deny the application until India has agreed to several stringent conditions. First, the Indian government should pledge not to construct further explosive devices. Second, the United States should exercise its option to buy back spent fuel already produced by the Tarapur reactor. Third, India should agree to place all its nuclear facilities under international safeguards. And fourth, India should agree not to reprocess any spent fuel, at least for the time being.

Clearly, the Indian government would not readily accept such conditions. But the opponents of the licence application point out that India would be hard pressed to find an alternative fuel supplier. The only other exporter of enriched uranium is the Soviet Union, and potential European exporters are at least ten years away from having significant export capacity. Thus, they argue that "if India wishes to avoid a shut-down of the Tarapur reactors, it may well have to deal with the United States, and the Commission has leverage to obtain non-proliferation ends".

But those views are not shared by the State Department. In a long statement delivered to the NRC last week, for example, Assistant Secretary of State Myron Kratzer argued that "the credibility of the United States as a reliable supplier of nuclear materials, equipment, and services is an essential ele-

ment in achievement of our non-proliferation objectives". Kratzer argued that the US-Indian agreement covering the Tarapur reactor is sufficient to prevent diversion of its plutonium to weapons production. And he warned that if the United States refuses to ship more fuel for the reactor, the Indian government could claim that the original agreement had been broken and that the spent fuel is therefore no longer under safeguards.

Kratzer announced, however, that the State Department is looking into the possibility of taking up the option to buy back the spent fuel which has already accumulated from the Tarapur reactor.

One key issue which surfaced during last week's hearings concerns the extent to which the United States provided aid for the production of India's first explosive device. The Cirus reactor, which provided plutonium for the device, was moderated by heavy water bought from the United States on

condition that it be used only for peaceful purposes.

When Senator Abraham Ribicoff last month drew attention to the possibility that American material had helped India produce its first atomic blast, the State Department demurred. It argued that, by the time the Cirus reactor began producing plutonium for the device, the US-supplied heavy water had been replaced with heavy water manufactured in India. Kratzer confirmed last week, however, that some US-supplied heavy water was probably still in the reactors at the time India used it to manufacture plutonium for the explosive. Consequently, critics of the proposed fuel sale argued that India cannot be trusted to abide by its pledges not to develop nuclear weapons.

The nub of this whole dispute is really that the opponents of the application are arguing that the time has come for the United States to make a public demonstration that it is serious

in its efforts to deter the spread of nuclear weapons, while the State Department is arguing that abrogation of the agreement to fuel Tarapur would jeopardise those US non-proliferation policies. The four NRC commissioners will be hard put to decide which side is correct.

At this stage, it's difficult to predict with certainty what the Commissioners will decide, but at least there is a clue. Earlier this year, there were two applications outstanding for exports of fuel for Tarapur—the 12,261 kg which was the subject of last week's hearings, and a separate shipment of 9,165 kg. Because at least one shipment was required urgently to avoid shutdown of the reactors, NRC approved the export of the smaller quantity on July 2. It did so by a vote of 3 to 1, but explicitly stated that the action would not prejudice its consideration of the second licence application. Its final decision will probably be made in the first week of August. □

IN BRIEF

Steвер's appointment

After weeks of uncertainty and delay, President Ford last week nominated Dr H. Guyford Steвер to be his science adviser and the first Director of the new White House Office of Science and Technology Policy (OSTP). Steвер's nomination must be confirmed by the Senate, but swift approval is expected. The appointment was delayed because four right-wing Republican Senators last month criticised Steвер's record as Director of the National Science Foundation and urged Ford not to nominate him for the White House post. The delay has ensured that OSTP will have little influence for several months. The Ford Administration's longevity is in considerable doubt, and with the election in full swing, few people will pay attention to the office. Nevertheless, President Ford can at least claim that he has restored science advice to the White House.

The axeman cometh

The £1,000 million public expenditure cuts announced by the UK Government last week, which are due to take effect in 1977-78, have not left science-related activities unscathed. With the axe falling on capital investment programmes of the nationalised industries, the energy sector (with the exception of the British National Oil Corporation) is particularly hard hit. Coal, gas and electricity will be seeking a total saving of about £70 million through deferred projects. Apart from the

Selby coal scheme, this may involve a 12-month postponement on the Steam Generating Heavy Water Reactor and a cutback on research and development of the fast breeder reactor. A cut of £100 million is being sought from the Ministry of Defence: most of the cuts will be achieved by removing or rephrasing existing programmes, and research work may not escape. At the Department of Education and Science the science budget will be cut in 1977-78 by £5 million. Details have yet to be made known, and it is thought that advice from the Advisory Board for the Research Councils will be sought before final decisions are made.

Geothermal energy research programme

A three-year programme of geothermal research in the UK is to be supported by the Department of Energy following the publication of a report, *Geothermal Energy: the case for research in the United Kingdom* (HMSO, £1.85). The report, by Dr J. D. Garnish of the Energy Technology Support Unit (ETSU), Harwell, reckons that likely returns warrant a modest research programme of collection and refinement of data.

Interest centres on two techniques—the extraction of hot water from aquifers in sedimentary basins or near springs, and the hydrofracturing of rocks, usually granite, with a higher than average temperature. Temperatures likely to be encountered with

either technique are in the 100 to 200 °C range.

Much of the £840,000 involved in the research programme (which ETSU will supervise) will go to the Institute of Geological Sciences for data gathering, particularly in Cornwall, Durham, Bath, Bristol, the Hampshire Basin and the Midland Valley of Scotland. Imperial College and Oxford University are also likely to receive support.

Plea for prisoners

Two scientists, Sergei Kovalyov (Soviet Union) and Sandor Arancibia (Chile) were the subject of appeals by a group of distinguished biologists on the occasion of international congresses of endocrinology and biochemistry in Hamburg recently. Kovalyov, an electrophysiologist, was sentenced in December 1975 to seven years in prison and three years exile for anti-Soviet activities. Arancibia, a neuroendocrinologist, was given a life sentence—he had been a prefect in the Valdivian region before the 1973 change of regime. Signatories included A. Lwoff, F. Jacob, J.-P. Changeux, F. Gros, F. Morel, Y. Laporte, E. Baulieu, J. Nunez, C. Kordon, C. B. Anfinsen, C. deDube, G. Wald, D. Baltimore, R. Dulbecco, S. Luria, A. Szent-Györgyi, J. D. Watson, H. Krebs, H. Temin, J. Axelrod, V. Ramirez, L. Martini and J.-P. Waller. They call on Mr Brezhnev and General Pinochet to free their colleagues strictly for humanitarian purposes since "their lives are in danger and those of their families are broken".

news and views

The history of the term 'ionosphere'

from C. S. Gillmor

FOR more than four decades the term "ionosphere" has been used to designate the ionised regions from about 60 kilometres to some thousands of kilometres above the Earth's surface. These regions were not always so named. In the first years after G. Marconi's demonstration in 1901 of trans-Atlantic radio communication, some pioneers of wireless, such as A. E. Kennelly, O. Heaviside and J. Erskine-Murray, designated the upper atmospheric region which supposedly played a part in long distance radio transmission as the "upper reflecting surface" or the "conducting layer". Many others, of course, believed no such ionised region existed. Discussion was stimulated by G. Marconi's lectures where he described the difference between day and night transmission (Marconi, *Proc. R. Soc. A.*, **70**, 344–347; 1902). Marconi termed the "day effect" (what would later be called the "night effect"), the phenomenon that radio transmission during the daytime seemed possible over a distance only two-fifths as great as that at night. Marconi's original explanation was that this was due to local effects of sunlight on the transmitting antennae. Others attributed the effect to increased ionisation of the upper atmosphere by the Sun.

The work of W. H. Eccles, L. de Forest, and L. F. Fuller just before World War I (Tuve, *J. atmos. terr. Phys.*, **36**, 2079–2083; 1974) stimulated a great debate on the nature and effective height of the upper region. Though Eccles suggested the name "Heaviside layer" in 1912 (Eccles, *Proc. R. Soc. A.*, **87**, 79–99, 1912), no single term emerged. L. W. Austin, O. Lodge, L. Cohen, G. W. O. Howe and others spoke of the "upper atmosphere", "upper conducting layer", "far upper regions", and "ionised upper atmosphere". Following World War I, Eccles' term was revived and "Heaviside layer" ("Kennelly-Heaviside layer" according to some), became the most frequently used term with "upper conducting layer" the next preferred.

By the early 1920s it was not so clear that "Heaviside layer" should be synonymous with the upper ionised regions of the atmosphere because various geophysical phenomena were known to occur at different heights in the upper atmosphere. A. Schuster called them the "upper conducting layers" (Schuster, *Electrician*, **89**, 325; Sept. 22, 1922). W. H. Eccles pointed out that the "night effect" was probably caused by the lower edge of the upper region of the atmosphere where free electrons "exist more or less permanently" (Eccles, *Electrician*, **94**, 208; Feb. 20, 1925). This lower edge is the Heaviside layer, he said, and it might be located higher or lower than the ionised region needed to explain the diurnal magnetic variations. Below this was the "middle atmosphere" where ions of molecular size are formed daily by sunlight. These ideas were not new, he said, but had been assumed long ago by students of wireless.

As a scientific specialty matures it concerns itself with standards of measurement, technique and nomenclature. This is evidenced by the actions of the British Radio Research Board (RRB), set up after World War I. In the autumn of 1921 the RRB Sub-Committee A, on Propagation of Wireless Waves, discussed (*RRB Sub-Committee A, Paper 27*; 1921) the recommendations of an international committee which had proposed standardising terms: "radio" in place of "wireless telegraphy"; "atmospherics" in place of "parasitics", "statics" or "X" (strays); "electronic tube" in place of "valve", "lamp" or "audion"; and "antenna" in place of "aerial". In the spring of 1924 the Sub-Committee discussed (*RRB Sub-Committee A, Paper 68*; March 8, 1924) terms for the classification of radio emissions (Continuous Waves (CW) and modulated forms of CW, and Damped Waves). It was probably at some similar meeting in the autumn of 1926 that the question was raised of agreeing on a term to designate the entire atmospheric region or regions

where ionised particles accounted for the major phenomena. In fact, R. A. Watson-Watt referred to recent discussions on nomenclature in a letter to the Secretary of the RRB. In this letter (Gardiner, *Nature*, **224**, 1096; 1969), dated November 8, 1926, Watson-Watt suggested the term "ionosphere" in place of what had been loosely termed the "upper conducting layer", and mentioned the analogy to stratosphere and troposphere.

Now it seems that E. V. Appleton was thinking similarly, for in a letter (*Edinburgh University Library, MS. Gen. 1985/21*) to J. A. Ratcliffe dated November 2, 1926 Appleton wrote:

"For the ionised part of the upper atmosphere I think the terms ionosphere or electrosphere might be useful. Which do you prefer? Cf. Stratosphere & Troposphere".

Watson-Watt and Appleton had numerous opportunities in those days for conversation at the Radio Research Station at Slough, at meetings of the RRB, and at King's College, London. In spite of the RRB discussions, "ionosphere" seems at first to have fared little better than the terms designed to replace "wireless" or "valve". The first to use the term "ionosphere" in print was Watson-Watt in 1929 in the Symond Memorial Lecture to the Royal Meteorological Society (Watson-Watt, *Q. Jl R. met. Soc.*, **55**, 273–301; 1929). He noted:

"It might be permissible to call it the Balfour-Stewart-Fitzgerald-Heaviside - Kennelly - Zenneck - Schuster-Eccles-Larmor-Appleton space, but something less direct is desirable. I have suggested the name ionosphere to make a systematic group troposphere, stratosphere, ionosphere but meanwhile the term 'upper conducting layers' seems to hold the field".

The term was next used, in its German form "ionosphäre", by H. Plendl who, independently, suggested it early in 1931 and used it in articles published

later that year (Plendl, *Jb. dt. Vers. Anst. Luftf.*, 665-671; 1931).

"Ionosphere" came into use widely beginning only in the period 1932-1934 when Watson-Watt, Appleton, Ratcliffe, W. Dieminger, I. Ranzi, D. F. Martyn, S. K. Mitra and others began to use the word in the titles of published papers and lectures. An example of its legitimisation was the prominent use of "Ionosphere" in the Kelvin Lecture delivered at the Institution of Electrical Engineers in April 1933 by Sir Frank Smith, Secretary of the Department of Scientific and Industrial Research (Smith, *Electrician*, **110**, 581-582; May 5, 1933). It was thus that the term began to appear in library indices and abstracting categories. It then began to be used in related

forms; the pulsing vertical incidence radar became known as the ionosonde, and ionospheric workers were sometimes known as ionosphericists. Use of the term was at its relative maximum during the early 1950s when about 35% of all published papers on ionospheric physics (Manning, *Bibliography of the Ionosphere*, Stanford University Press, Stanford, California, 1962) had the word ionosphere or a form of it in their titles. This trend has reversed as other specialties have developed out of ionospheric physics and terms such as "magnetosphere" and "STP" (solar-terrestrial-physics) have come into common use to designate many subjects which formerly were included primarily within the specialty of ionospheric physics. □

number of human carcinomas show that some cells grow in suspension and fail to form tumours in nude mice, some anchorage-dependent lines are tumorigenic and furthermore there is no correlation between the ability to activate plasmin and either growth in suspension or tumorigenicity (Marshall, Franks and Carbonell, personal communication; Pearlstein *et al.*, *Cancer Res.*, **36**, 1575; 1976; Jones *et al.*, *Int. J. Cancer*, **16**, 616; 1975).

These observations raise several questions not least of which is the suitability of the commonly used 'fibroblast' cells and their viral transformants as suitable models for real cancer, but also the use of the nude mouse as a means of detecting tumorigenicity.

There have been mutterings about established cell lines such as 3T3 for some time, especially because of their heteroploid karyotype. It is now clear, however, that the BALB/c3T3 cell line is frankly tumorigenic when attached to glass beads and inoculated into suitable hosts (Boone, *Science*, **188**, 68; 1975). Moreover, the tumours formed are 'vasoformative sarcomas' (Boone *et al.*, *Cancer Res.*, **36**, 1626; 1976) so it seems that these cells are not fibroblasts but arise from endothelial tissue. In fact, a great many cell lines established from a wide variety of tissues appear to be derived from the endothelial cells and pericytes of small blood vessels (Franks and Wilson, *Eur. J. Cancer*, **6**, 517; 1970), so many of the different established cell lines may represent a very restricted sample of the variety of cell types in the body.

Studies of the incidence with age of a great many naturally occurring cancers in man lend support to the hypothesis that cancer cells arise after a small number of heritable changes have occurred in a normal cell. Presumably the altered cell becomes a successful cancer if these changes result in it being better able to proliferate than the cell type it comes from. There is no reason to suppose that the changes which confer a selective advantage on one cell type in one environment will confer selective advantage on another, nor even that selective advantage within a given tissue need arise through a unique series of steps; other pathways could be equally effective. As a consequence tumour cells from widely different tissues might be expected to have widely different properties when studied *in vitro*. After all what is good for the endothelial cell may not be so good for the bladder.

Such generalisations that have been drawn about the nature of neoplastic change from the use of cultured cells may simply reflect the small number of cell types examined and the even smaller number of oncogenic agents.

Transformation and tumorigenicity

from Robert Shields

SOME recent observations on the properties of cells transformed by mammalian tumour viruses and their ability to cause tumours when injected into the commonly used nude mouse, raise several questions on the validity of treating these experimental systems as a model for real cancers.

Ever since the first mammalian cell cultures were transformed by tumour viruses a great deal of effort has been expended in attempts to understand the process of oncogenic change *in vitro*. Studies using a few cell types and even fewer viruses have collected an enormous number of *in vitro* cellular properties that are often associated with tumorigenicity. These include changes in cellular morphology, increased cell autonomy, loss of density-dependent inhibition of growth, decreased dependence on high levels of serum in the culture fluid, increased agglutinability by plant lectins, changes in membrane composition, increased ability to grow in gel suspension (loss of anchorage dependence) and so on and so on. What is a pity is that much of the work on these properties has been performed on established lines of virally transformed cells so it has been impossible to unravel which changes are caused by the virus and which have arisen through selection in culture.

One way around this problem has been to select at random clones of 3T3 or primary rat embryo cells recently infected with SV40 and examine each clone for various parameters of transformation (Risser and Pollack, *Virology*, **59**, 477, 1974; Risser, Rifkin, and Pollack, *Cold Spring Harbor Symp. quant. Biol.*, **39**, 317; 1974). In this way it was hoped to find which parameters of transformed cells are related to the

viral gene, which changes are related to each other, and which changes confer tumorigenicity.

The results from these experiments showed that the virus was able to elicit a whole spectrum of changes. The only alteration in some clones of 3T3 cells was that they could grow in low concentrations of serum, and that they could grow to higher cell densities. These cells were negative for the virus-specified T antigen, no virus could be rescued from them and the cells could be fully transformed by reinfection with SV40. All this suggests that these changes may be caused by a hit and run action of the virus rather than by the continued presence of the viral gene.

The only *in vitro* cellular property that correlated with the ability of the cells to cause tumours in nude mice (which lack cell-mediated immunity), was their ability to grow in methocel suspension (anchorage independence) (Shin *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **72**, 4435; 1975) and this property was in turn correlated with the ability of the cells to produce a protease that converts plasminogen in the serum to plasmin (Pollack *et al.*, *Proc. natn. Acad. Sci. U.S.A.*, **71**, 4792; 1974). These experiments were performed with only two cell types and one transforming agent but many other reports have suggested a correlation between anchorage-independent growth and tumorigenicity (Freedman and Shin, *Cell*, **3**, 355; 1974; Laug *et al.*, *J. natn. Cancer Inst.*, **54**, 173; 1975).

The correlation between growth in suspension and tumorigenicity is far from absolute, however. Examination of epithelial cell lines derived from a

Mars from Viking 1

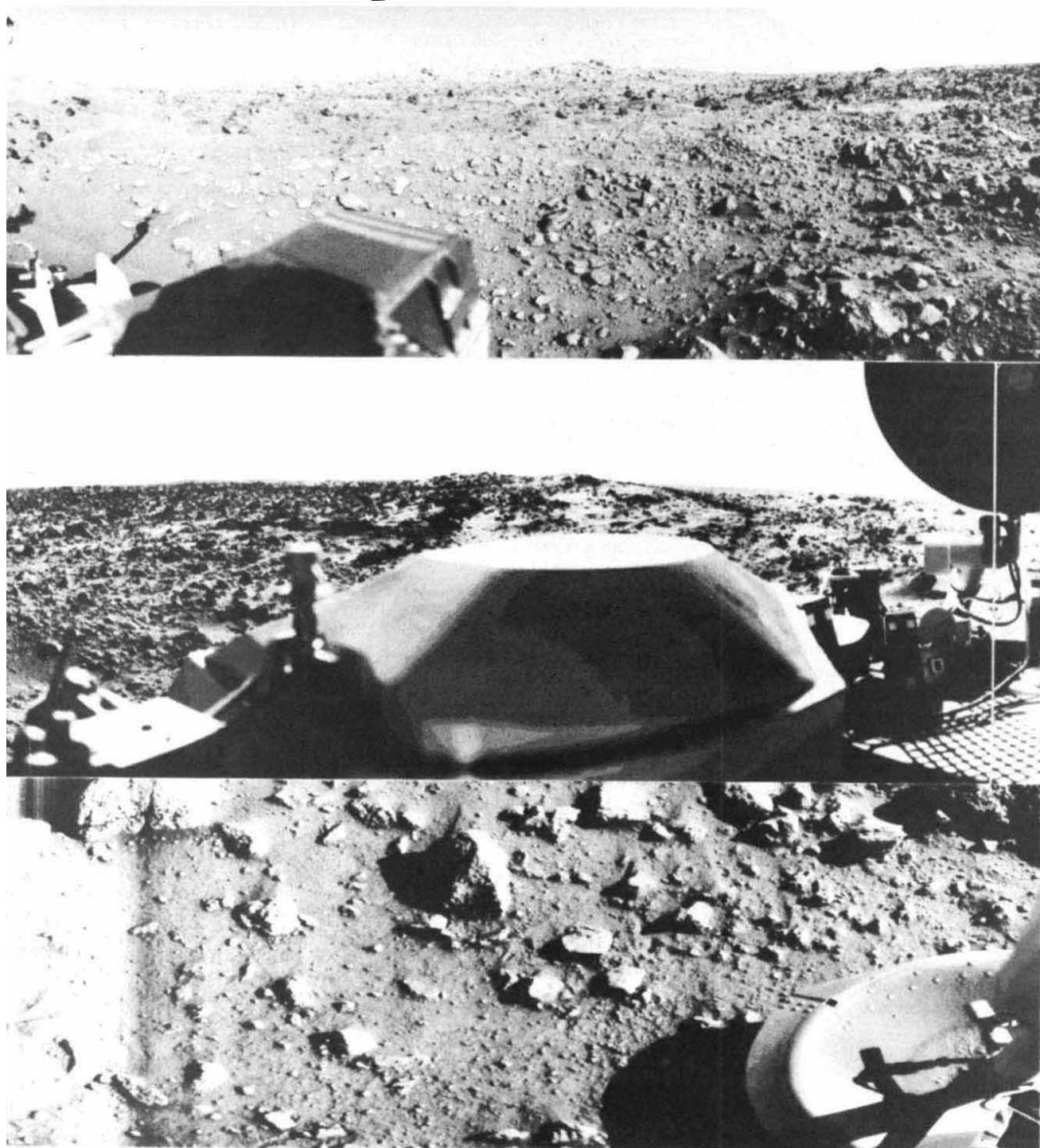


Photo: NASA

THE top two pictures provide the first panorama of the Martian landscape as seen from Viking 1, taken in the late Martian afternoon. A plateau can be seen on the horizon to the left of the top picture, approximately 3 kilometres (1.8 miles) away. The out-of-focus spacecraft component left of centre is the housing for the sample arm (which is now in full working order after becoming jammed when first tested). The centre photograph is the continuation to the right of the 300° panorama. At left is the low-gain antenna which receives commands from Earth. In the right foreground

are colour charts for lander camera calibration, a mirror for the Viking magnetic properties experiment and part of a grid on the top of the lander body. At upper right is the high-gain dish antenna for direct communication between the landed spacecraft and Earth. Toward the right edge is an array of smooth fine-grained material which shows some hint of ripple structure and may be the beginning of a large dune field off to the right of the picture.

The bottom picture, the first ever of the surface of Mars, was taken minutes after the spacecraft landed.

Do exploding stars leave tracks in the dust?

from M. G. Edmunds

HAVE grains of dust, formed in the explosion of stars, managed to survive incorporation into Solar System meteorites? A most interesting observation, although unfortunately open to rather ambiguous theoretical interpretation, has recently been reported by J. Audouze and co-workers (*Astrophys. J. Lett.* **206**, L185; 1976).

They examined micron-sized grains extracted from a separated part of the Orgueil meteorite. The material from which these grains came shows an unusual chemical composition in that the abundance of ^{22}Ne , compared with other neon isotopes, is much higher than in most meteoritic material. The unusual isotope ratios are not consistent with either trapping of neon from the solar wind, or with creation of neon by nuclear reactions involving the impact of high-energy cosmic ray particles. The origin of the excess ^{22}Ne remains disputed.

Audouze *et al.* use a technique of chemically etching the radiation damage tracks left by the passage of energetic heavy ions through the grains. The etched tracks can be examined with a high voltage electron microscope. They found tracks in those grains which appeared amorphous and rounded, and the density of tracks implies that these grains have been strongly irradiated at some time. Audouze *et al.* take the view that the irradiating particles were probably heavy ions with masses comparable to iron nuclei. An experimental exposure to a beam of iron nuclei caused crystalline grains (which did not initially have tracks) to show tracks similar to those found in the amorphous grains. By assuming the currently observed ratio of heavy ions to protons in cosmic rays and particles from solar flares, a net proton irradiation can be estimated for the amorphous grains.

One interpretation of the ^{22}Ne anomaly is offered by production from spallation nuclear reaction of the irradiating protons on ^{24}Mg , but this requires that the energy spectrum of the protons has a rather peculiar "pulse" shape to avoid overproduction of the other neon isotopes. A high flux of particles might have occurred during the early history of the Solar System, but the observed irradiation would imply a very much higher flux of particles from the Sun at early times compared with that observed today.

A second interpretation is perhaps more interesting. It may be possible for

grains to condense in the expanding envelope of gas generated by the supernova or nova explosion of a star. In a series of papers (the most recent being Clayton and Hoyle, *Astrophys. J.*, **203**, 490; 1976) it has been suggested that radioactive atoms from the explosive nucleosynthesis occurring in novae or supernovae could become trapped in the grains as they condense. If these grains could subsequently survive the physical conditions during the formation of the Solar System and be incorporated into meteorites, then the products of radioactive decay of the nuclei trapped in the grains could show up as isotopic anomalies. The excess ^{22}Ne for example might come from the decay of ^{22}Na produced in explosive nucleosynthesis, and either absorbed by grains as ^{22}Ne , or incorporated as ^{22}Na which later decays. The evidence of high irradiation of the meteoritic grains could just imply that some do indeed come from the violent environment of novae and supernovae where high particle fluxes are expected. The particle flux could also provide some additional ^{22}Ne by spallation reactions.

It has usually been assumed that the meteoritic isotope ratios reflect conditions in the early Solar System. There exists a real difficulty in accepting that grains from novae or supernovae survive into meteorites. Such grains would bring other isotopic anomalies with them which would upset the elegant inferences about the timescales involved in the formation of the Solar System drawn from isotope ratios (so-called "nucleocosmochronology"). But there are plenty of isotopic anomalies, and a surprising lack of them for certain elements, to keep discussion alive before a revision of timescales is accepted. Audouze *et al.*'s paper at least indicates that violent irradiation of grains existing when the meteorites formed did occur somewhere, and has to be explained. □

HLA and disease

from G. Nicholas Rogentine Jr

The First International Symposium on HLA and disease was held in Paris on June 23–25. It was organised by J. Dausset and A. Svejgaard. The proceedings will be published as a book.

BASIC and clinical scientists gathered in Paris to try to understand the meaning of the association of HLA antigens with disease. While the meeting did not fully achieve this goal, it did estab-

lish clearly that many highly significant HLA antigen frequency perturbations exist in a remarkable variety of human diseases.

HLA antigens are products of genes at four closely linked loci on the sixth chromosome. HLA-A, HLA-B and HLA-C antigens are identified serologically on lymphocytes. These antigens are found on most cell membranes. Products of the fourth locus, HLA-D, are defined by the mixed leukocyte culture reaction; these products or closely related ones also seem to be B-lymphocyte alloantigens.

Most of the exciting new data was presented in the workshops, organised according to disease classification. Well established associations were confirmed and many new correlations were reported. The clearest, all highly significant and involving increases (suggesting a susceptibility to disease) in antigen frequency, were the following: HLA-B27, ankylosing spondylitis, Reiter's syndrome and acute anterior uveitis; HLA-Dw4, rheumatoid arthritis; HLA-Dw2 and HLA-B7, multiple sclerosis; HLA-Dw3 and/or HLA-B8, gluten-sensitive enteropathy, dermatitis herpetiformis, myasthenia gravis (in young females without thyomas), juvenile diabetes, Graves' disease, chronic active hepatitis and Sjögren's syndrome; HLA-B5, Behcet's disease; HLA-A3, idiopathic haemochromatosis; HLA-A2, childhood acute lymphocytic leukaemia; HLA-A2 and SIN2, nasopharyngeal carcinoma in Chinese; HLA-A1, Hodgkin's disease; HLA-Bw40, oesophageal carcinoma in Northern Iranians. In Orientals, who as a race do not have HLA-B8, myasthenia gravis and Graves' disease were associated with HLA-B12 and HLA-Bw35, respectively. Nearly all patients with gluten-sensitive enteropathy and dermatitis herpetiformis are also positive for a B-lymphocyte alloantigen not linked to HLA.

A strong theme unrelated to disease susceptibility emerged from the workshop on malignant diseases: the possession of certain HLA antigens may be associated with long term survival. This has been seen in acute lymphocytic and acute myelocytic leukaemia, Hodgkin's disease, and bronchogenic carcinoma. These observations need confirmation by large scale prospective studies.

What do these associations mean pathophysiologically? As suggested by H. McDavitt (Palo Alto), many correlations could be explained by invoking a pathogenically active immune response gene in linkage disequilibrium with the disease-associated HLA gene, the latter being merely a marker. Linkage disequilibrium is known to occur all along the HLA region and, by analogy with mice, immune response

genes probably reside in this area, too. But proof of a human immune response gene does not exist yet. R. Zinkernagel (La Jolla) and G. Shearer (Bethesda) suggested that HLA antigens are directly involved in the disease process by analogy with their findings with mice—the “altered self” hypothesis, in which histocompatibility antigens are modified by viruses or other substances rendering them susceptible to attack by autologous T lymphocytes. A. Sveigaard (Copenhagen) speculated that HLA antigens might competitively inhibit binding of various non-immune ligands, hormones, for example, and in this way provoke disease. F. Lilly's (New York) work with murine Friend virus leukaemogenesis suggests that some D-region H-2 antigens are receptors for leukaemia virus and thus participate directly in disease susceptibility. The mouse D region seems to be analogous with the human HLA-A locus. This locus is implicated in leukaemia, Hodgkin's disease and nasopharyngeal carcinoma.

Although no definite pathophysiological links between HLA and disease have been found, in at least two disorders—gluten-sensitive enteropathy and chronic active hepatitis—they may be discovered soon. Both these diseases have known causal agents and disordered immunity. Both are HLA-Dw3 or HLA-B8-associated.

J. Dausset (Paris) emphasised several areas where these associations might be clinically useful. One interesting area is preventative medicine and early diagnosis. Any HLA-disease association necessarily implies a greater risk of developing the disease in a person who has that particular antigen. The relative risk for developing ankylosing spondylitis in HLA-B27 positive Caucasian males is about 90 times that of HLA-B27 negative males. Although most HLA-B27 positives do not develop this disease, nevertheless, they are more likely to do so than the B27 negatives. Knowing these risk factors can help focus awareness on the kinds of diseases most likely to affect each individual during his lifetime. It is not too far-fetched to expect that establishing HLA types will become an integral part of preventative medicine and health maintenance in the future. □

r, K and evolution

from Peter D. Moore

THERE is a taxonomic imperative in man which makes him delight in pigeon holes and simple labels. One of its most recent products in the ecological field has been the concept of *r* and *K* selection, whereby organisms can be classified along a con-



A hundred years ago

LIEUT. CHRISTIE, R. E., writing to us from Madras with regard to the use of selenium in telegraphy, says that if we could do away with the man (or woman) signaller, and substitute a commutator actuated by a current of electricity generated by the action of light upon a piece of selenium, we should (supposing the sensitiveness of the selenium to be adequate) have a combination capable of enormously increased rapidity. The message to be transmitted would be first set up (by mechanical means) in the Morse character, in long and short *slits* in an opaque screen; and this perforated screen being passed rapidly between the selenium and a source of light, the currents of electricity would be generated which are required for actuating the commutator. The possibility of such a combination depends on the sensitiveness of selenium to the influence of light. Assuming the combination to be possible, the rapidity of signalling would seem to be limited only by either the mechanical conditions of the commutator (or relay), or the power of the printing instrument at the receiving station.

THE Vienna earthquake, to which we referred last week, occurred on July 17 at 1.22 p.m. The principal seat of the commotion was Scheibbs, a small country place forty miles west of Vienna; almost every house in Scheibbs has been damaged. The area of the commotion was very large, equal to about two-thirds of England. It reached Austria proper, Moravia, part of Bohemia, and Hungary. The last earthquake in Vienna was on January 3, 1873. Fifteen instances of earthquake have been recorded in Vienna from the beginning of the thirteenth to the end of the eighteenth century. None of them produced any real damage, except those of September, 1590, and December 4, 1689.

from *Nature*, 14, July 27, 279; 1876.

tinuum according to the proportion of their energy expended on reproduction. A high proportion results in a high initial rate of population increase (*r* in the logistic equation describing population growth). In unstable, unpredictable habitats, selective pressures operate in favour of this type of organism, whereas such taxa are at no advantage in stable environments.

Within many groups of organisms it is possible to recognise species which tend towards one end or other of the *r*-*K* continuum. For example, Southward *et al.* (*Am. Nat.*, 108, 791; 1974) have considered its application to birds and Lepidoptera, and come to the conclusion that *r*-selected organisms are more likely sources of evolution and speciation than are *K*-selected organisms. Some rapidly evolving plant

taxa even seem to have an *r*-*K* spectrum exhibited within a species or microspecies cluster, for example, in dandelions (Solbrig and Simpson, *J. Ecol.*, 62, 473; 1974) and in sunflowers (Gaines *et al.*, *Am. Nat.*, 108, 889; 1974).

Two further examples of this evolutionary process have now come to light, the kidney vetch (*Anthyllis vulneraria*) in Europe and the bigberry manzanita (*Arctostaphylos glauca*) in North America. Sterk (*Acta. bot. neerl.*, 24, 315; 1975) has examined the population dynamics of coastal and inland populations of *Anthyllis* in the Netherlands. In the open, unstable, coastal sites the population density fluctuated from year to year. Mortality seemed to be related to stresses in the abiotic environment, such as drought, whereas mortality in the inland, stable sites was the outcome of competitive interplay. In the inland sites, fewer flowers were produced per plant than at the coast and flowering began when the plants were older. The data seem to conform with the idea of a relative *r* and *K* selection being operative respectively at the coast and inland sites.

Vasek and Clovis (*Am. J. Bot.*, 63, 189; 1976) describe two ecotypes of *Arctostaphylos glauca*, one an upright shrub of the chaparral, and the other a low, spreading plant of desert pinyon woodland. Apart from these morphological differences, the two ecotypes differ in their flowering behaviour. The chaparral ecotype produces almost double the number of flowers, is more seasonally constrained in its flowering, and the total amount of fruit produced is about five times that of the desert ecotype. The morphological adaptations of these plants can be related to climatic conditions; fruiting potential may be a consequence of disturbance of the chaparral by frequent fires. In the more stable desert community, vegetative reproduction (by layering) becomes possible and seed production is therefore of lesser importance. Once again it can be argued that these ecotypes represent a genetic divergence in response to *r* and *K* selection in these habitats, but in this case the contribution of vegetative spread to the reproductive capacity of the “*K*-selected” ecotype complicates the issue. Seed production is easily measured so it is tempting to use this as an index of a plant's capacity to expand its population rapidly (high ‘*r*’). But in many plant species this represents only a portion of the total energy resource devoted to reproduction.

If the selective pressures of stable environments always favour a reduction in seed output, how does one explain the figures of Salisbury (*Proc. R. Soc. Lond.*, B188, 183; 1975, and 192, 323; 1976) concerning seed pro-

duction in plants? Within a single habitat, such as calcareous grassland, there is a considerable range of seed production per plant, for example, *Thesium humifusum*, produces 100 per plant; *Polygala calcarea*, 270; *Carlina vulgaris*, 680; *Anacamptis pyramidalis*, 35,000; *Senecio jacobaea*, 50,000; *Orchis maculata*, 56,000 and *Orobancha elatior*, 98,000. As Salisbury points out, these figures relate most closely to the relative risks experienced with different mechanisms of seed dispersal, wind being more risky (and therefore more wasteful) than animal dispersal.

Salisbury (1976) quotes *Juncus effusus* and *J. tenuis* as an example of contrasting seed production resulting from the use of different dispersal mechanisms. *J. effusus* produces between 1.5 and 3 million seeds per plant (wind dispersed) whereas *J. tenuis* produces 3,000–4,000 (adhesion to animals and vehicles). Thus two species of the same genus, both occupying unstable habitats, in the successional sense, differ in their seed production by three orders of magnitude.

Evidently there are several evolutionary answers to any environmental problem. In a given set of conditions, the output of seed from a taxon is influenced by such factors as the relative importance of vegetative reproduction and the seed dispersal mechanism used, as well as any response to the stability (r - K) gradient. The simplest answers may be the most attractive, but they are not necessarily the most complete ones. □

Chemistry does not define environment

from Peter J. Smith

In the past, many workers have placed great reliance on chemical characteristics as indicators of the geological, and especially crustal, environments in which Archean basalts were emplaced. Some, for example, have seen the lack of crustal contamination observed in low-potassium tholeiites in greenstone belts as an important argument against the presence of sialic crust beneath the successions. Others have regarded the calc-alkaline affinities of certain greenstone basalts as evidence of an original island arc environment. According to Gill and Bridgwater (*Earth planet. Sci. Lett.*, **29**, 276; 1976), however, the use of chemical comparisons between modern basalts and Archean basic rocks to identify ancient environments must now be regarded as a questionable procedure.

Gill and Bridgwater base their case on a study of the Amlalik dykes of West Greenland. This heterogeneous group of metamorphosed minor basic

Aggression in sticklebacks

from John Krebs

THE question of how different types of aggression in animals are related to one another has been a source of continual controversy. Aggressive behaviour in natural conditions is usually seen in competitive social encounters between members of the same species, for example in territorial defence and dominance disputes. On the other hand many experimental laboratory studies have been concerned with attacks directed at inanimate objects or at members of another species (for example a rat attacking a mouse). There have been few attempts to examine whether or not aggression in these different contexts depends on a common internal mechanism.

In a recent study F. A. Huntingford (*Anim. Behav.*, **24**, 245–260; 1976) has examined this question. Using that traditional ethological beast, the three-spined stickleback, she carried out a series of experiments to test whether individual males which were highly aggressive in one context would also score high in other situations. It has long been known that a male stickleback will vigorously attack a 'rival' male which is presented to him near his nest in a glass tube. Huntingford used this technique to compare the aggressive response, measured by biting and lunging, to a rival male, a male of the closely related but

quite distinct ten-spined stickleback, and a female smooth newt (a common inhabitant of the streams favoured by sticklebacks). Briefly, she found that the aggression scores for individual males to all three stimuli were well correlated. In two further experiments, Huntingford measured the reaction of the same group of males to a predator (a pike) and to being placed in an unfamiliar tank. She analysed these results by Principle Components Analysis and was able to identify a group of behaviours which could be classified as indicating 'boldness'; these included approaching the pike and showing normal swimming movements. Individuals which had scored high on the aggression tests also tended to be bold in the face of a predator and in an unfamiliar tank, and this was not simply a result of higher overall activity.

These results suggest that territorial aggression and aggression against predators may depend in part on common internal factors. Huntingford points out that this makes evolutionary sense, because stickleback populations exposed to heavy predation should not only tend to become timid in the presence of a predator, but also make themselves inconspicuous by not engaging in vigorous territorial displays.

intrusions, emplaced in 3,700–3,600 Myr old gneisses forming part of the western edge of the North Atlantic craton, has been affected variably by subsequent magmatic and tectonic events which enable a lower limit of 3,040 Myr to be placed on the dykes' ages. The total area of older sialic crust cut by the dykes exceeds 12,000 km², although there is no *a priori* reason for supposing all the dykes to have been emplaced in a single episode nor any for supposing all the dykes to have been derived from the same parent magma.

And the chemistry of the dykes suggests that any such suppositions would indeed be wrong. Although all the dykes studied were basaltic, their compositions ranged from that of primitive basalts reminiscent of modern oceanic basalts to more differentiated types akin to iron-rich tholeiites of modern continental flood basalt provinces. Some of this variation is undoubtedly due to subsequent metasomatism; but Gill and Bridgwater conclude from Rare Earth Element patterns, Ni, Sr, Ti and Zr contents and other chemical

indicators that much of it reflects a range of primary compositions.

If this is so, the dilemma is clear. The ridge-like Amlalik basalts, for example, were evidently not intruded into early ocean floor, although had they not equally evidently been intruded into granite gneiss their chemistry could have been taken incorrectly to indicate an oceanic origin. Moreover, these same ridge-like basalts are comparable in composition to greenstone successions in South Africa and Australia and some greenstone belts in the Canadian Shield. It is most reasonable, claim Gill and Bridgwater, to assume that the primary compositional variations represent differences in the depth of melting in the mantle and/or the speed at which the magmas rose to the surface. In other words, the variations reflect changes in the melting régime and/or degree of differentiation rather than differences in crustal environment (oceanic, continental, island arc). Ergo, inferences about crustal environment drawn from chemistry alone must be suspect. □

review article

Analysis of erythropoiesis at the molecular level

P. R. Harrison*

Recent work has clarified how different erythroid functions may be regulated during normal erythropoiesis, and have helped to define the role of the hormone erythropoietin. Studies with cell lines (Friend cells) capable of undergoing erythroid differentiation in culture have made possible a comparison of normal and leukaemic erythropoiesis, by the combined approaches of cell biology, molecular biology and somatic cell genetics.

Normal erythroid systems

ERYTHROPOIESIS has been recognised for many years as a useful model system for investigating how functions characteristic of a specialised differentiated cell are regulated in a coordinate manner within a single cell lineage. It is well established that erythroid cells as well as other blood cell types (almost certainly also including the lymphocyte population) are derived from a common haematopoietic stem cell (CFU-S, Fig. 1), and that commitment of CFU-S cells to a particular haematopoietic lineage is influenced by the microenvironment of particular haematopoietic organs. An elegant demonstration of the importance of such microenvironmental factors in commitment is in the classical experiments by Trentin and co-workers¹ which show that the type of haematopoietic cells formed (granulocytic or erythroid) changes dramatically in colonies growing across the junction of a plug of marrow grafted into spleen tissue. The existence of the Steel (SI) mutant in mice also demonstrates how a specific genetic locus can govern microenvironmental function in haematopoietic stem cell proliferation and differentiation, particularly affecting the erythroid lineage^{2,3}.

After commitment to the erythroid lineage, erythroid stem cells seem to proliferate for a few generations before becoming sensitive to the hormone erythropoietin⁴. An age structure therefore exists within the committed erythroid stem cell population; this is further demonstrated by recent techniques in which erythroid precursor cells are cloned *in vitro*⁵⁻⁷. In the presence of erythropoietin, two types of colony are produced: the first appears within 2 d of culture and consists of small differentiated erythroid colonies (about 60 cells) which are presumed to arise from late committed erythroid stem cells (CFU-E, Fig. 1) of limited proliferative potential^{5,6}. The second type of colony appears later (after about 10 d of culture) but comprises very large numbers (about 10⁴) of differentiated erythroid cells^{7,8}. These colonies are thought to arise by extensive proliferation of stem cells isolated soon after commitment to the erythroid line (BFU-E, Fig. 1).

Various groups of workers have attempted to analyse the regulatory mechanisms governing how committed erythroid stem cells differentiate to mature red blood cells, using various normal erythropoietic systems, for example, foetal liver during the hepatic phase of erythropoiesis. Recent work now seems to have established beyond reasonable

doubt that foetal erythropoiesis is initiated by haematopoietic stem cells migrating from the yolk sac⁹, rather than by stem cells originating *in situ* by inductive effects within the foetal liver itself¹⁰. CFU-S stem cells reach maximum numbers in foetal liver from day 14 of gestation¹¹, whereas stem cells committed to the erythroid line reach a peak at the days 15-16 of gestation (that is at the time of maximum production of red blood cells), after which they decline rapidly².

For many years, several groups have sought to analyse the process of erythroid differentiation in molecular terms, using *in vitro* cultures of erythroid cells. It is now generally accepted that erythropoietin acts primarily by increasing the proliferation of committed erythroid stem cells and proerythroblasts which then differentiate into mature erythroid cells containing haemoglobin¹²⁻¹⁴. More recent work by groups at the Beatson Institute and Columbia University (Marks, Rifkind and coworkers) has been concerned with identifying the stage at which the globin genes become active during erythroid development and whether erythropoietin has any effect on the timing of these events. At the Beatson Institute we have tried to elucidate this question by determining the earliest erythroid precursor cell in which globin messenger RNA accumulates, using a novel autoradiographic procedure¹⁵ for detecting cytoplasmic globin messenger RNA in fixed preparations of cells by *in situ* hybridisation with globin cDNA (that is, a highly radioactive transcript of globin messenger RNA prepared using reverse transcriptase). These studies show that globin messenger RNAs accumulate at the basophilic erythroblast stage (Fig. 1), but are not present in significant amounts in proerythroblasts from early foetal livers¹⁶. This conclusion is also consistent with conventional hybridisation studies¹⁶ with immature erythroid cells obtained from total foetal liver cells by removing mature cells by immune lysis using an antiserum raised against erythrocytes^{17,18}. Subsequent studies by the Beatson group have shown that elevated levels of erythropoietin enhance the accumulation of globin messenger RNAs in proerythroblasts, both in developing foetal livers *in vivo* or in culture *in vitro*, and in spleens of anaemic adult mice¹⁹. As suggested by Clissold²⁰, this result may explain her finding that immature erythroblasts isolated from severely anaemic rabbits, but not normal rabbits, are able to differentiate in culture without erythropoietin. This effect of elevated erythropoietin levels can have the result that globin messenger RNA and subsequently haemoglobin accumulate earlier than normal relative to other morpho-

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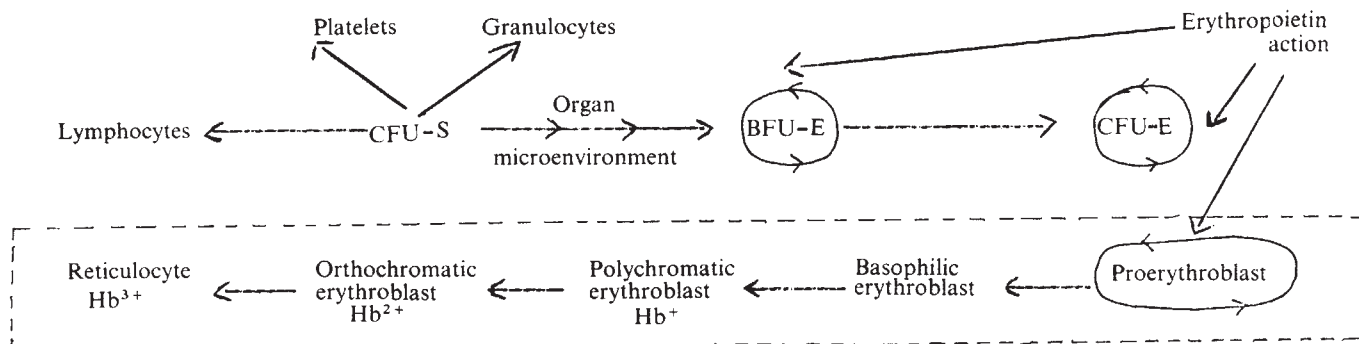


Fig. 1 Outline of formation of erythroid cells. Hb^+ – Hb^{3+} cells containing increasing amounts of haemoglobin. The cell types in the dotted box represent the recognisable erythroid series.

logical criteria, so that an atypical series of macroerythroblasts may be formed^{13,14} similar to that found in certain megaloblastic anaemias²¹.

Independently, the Columbia group has arrived at basically similar conclusions. Immature erythroid precursor cells (80–90% proerythroblasts²²) from foetal liver cells isolated by the immune-lysis procedure, contain negligible globin messenger RNAs as assayed either by translation in the Krebs ascites system²³ or by hybridisation with globin cDNA²⁴. After culture of such cells for 10 or 22 h, however, the cellular level of globin messenger RNAs increases 5 or 100 times²⁴ during which time most of the cells differentiate to the basophilic erythroblast stage²⁵ and begin to synthesise globin chains²³, although haemoglobin is not detectable cytochemically until 44 h of culture²⁴. These studies, therefore, also support the hypothesis that globin messenger RNA accumulation is enhanced between the proerythroblast and basophilic erythroblast stages of development; and further show that there is no pool of untranslatable globin messenger RNA in immature erythroid cells. However, the Columbia group find that treatment of cultures with erythropoietin does not increase the average cellular content of globin messenger RNA; although the number of globin messenger RNA-containing cells is greatly increased²⁴. The explanation for this discrepancy may be in the doses of erythropoietin, or the degree of anaemia, involved in the two series of experiments.

The Friend cell system

Studies with normal erythropoietic systems are limited by the difficulties of obtaining large numbers of erythroid cells at specific stages of development, the short term nature of the *in vitro* culture systems, and the impossibility of performing genetic studies. The discovery by Dr Charlotte Friend of tissue culture cell lines which are capable of undergoing erythroid differentiation has therefore opened up new possibilities.

Friend cells were obtained by culturing fragments of spleens from mice infected with the Friend virus complex²⁵. The nature and host control of Friend virus infection have already been reviewed in considerable detail^{26,27} and will not be further considered here in spite of their importance and relevance. In interpreting any studies with Friend cells, it is, however, important to be clear about their relationship to normal erythroid precursor cells. Although this is still to some extent a subject of controversy²⁸, it seems most probable that the target cell for Friend virus is a late committed erythroid stem cell (CFU-E, Fig. 1). Two recent and compelling lines of evidence favour this hypothesis. First, the elimination of multipotential, but not erythroid committed stem cells, does not eliminate Friend virus-induced proliferation of erythroid cells in the spleens of polycythaemic mice; whereas no response to Friend virus is observed in mice in which the committed erythroid cell compartment has been depressed²⁹. Second, inoculation of

lethally irradiated mice with normal spleen cells gives rise to erythroid, granulocytic and megakaryocytic colonies in the spleen; however, infection of such mice with Friend virus four days after grafting yields in addition hyperbasophilic (Friend cell) colonies solely within the differentiated erythroid colonies³⁰, although all types of haematopoietic colonies in the spleen are known to contain multi-potential stem cells¹. Thus, although the number of non-erythroid cell types may also be affected³¹, Friend virus seems to have specific effects on committed erythroid cells, rendering them capable of proliferation and differentiation independently of the hormone erythropoietin^{32–34}. Friend cell lines established in culture seem to be transformed erythroid precursor cells selected by the culture conditions (or otherwise) which are arrested at the proerythroblast stage of development²⁵, and are not responsive to erythropoietin^{35–37}. In this respect, Friend cells are therefore quite different from normal erythroid precursor cells. In fact, there is one report that Friend cells grown *in vivo* or in plasma clots *in vitro* do show manifestations of granulocytic differentiation³⁸, but this remains to be substantiated.

On treatment with dimethylsulphoxide (DMSO)³⁹ or a range of other chemical agents^{40–43}, Friend cells undergo a series of changes many of which are characteristics of normal erythroid cell differentiation; for example: chromatin condensation and other morphological changes resulting in a non-dividing cell³⁹; accumulation of globin messenger RNAs^{44–46} and strain-specific globins^{47,48}, increase in δ -aminolaevulinic acid synthetase activity⁴⁹ and haem synthesis⁵⁰; erythrocyte-specific membrane changes^{50,51} and an increase in the major erythrocyte-specific membrane protein (H. Eisen, personal communication); and an increase in carbonic anhydrase activity⁵². Changes in histone F2A2 subfractions also occur⁵³. Thus, although Friend cells are abnormal in their dependence of the hormone erythropoietin, in certain conditions they do exhibit many characteristics of normal erythropoiesis.

Recently, Orkin *et al.*⁵⁴ have shown that during treatment of Friend cells with DMSO the ratio of α to β globin mRNAs decreases from about 3.5 to about 1.0, the value characteristic of the normal erythrocyte. Our own hybridisation studies performed with purified normal erythroid precursor cells¹⁶, and also those of the Columbia University group²⁴, suggest, but do not prove definitively, that this differential timing of expression of the α and β globin genes is not a characteristic of normal erythroid cell development.

The mechanism whereby the various inducers of erythroid differentiation operate is not clear. Sieber⁵⁵ has made the interesting observation that DMSO enhances the sensitivity of normal CFU-Es, but not BFU-Es, to non-saturating doses of erythropoietin. This result may be related to the finding that Friend cells seem to be marginally sensitive to erythropoietin after DMSO treatment³⁵. Certainly, membrane function seems to be involved since local anaesthetics prevent the action of all inducers of erythroid differentiation

so far tested without affecting proliferation of Friend cells⁵⁶ *per se*.

Several circumstantial lines of evidence suggest that induction of differentiation in Friend cells requires some cell-cycle dependent process. McClintock and Papaconstantinou⁵⁷ have observed a correlation between the cell doubling time and the time of onset of haemoglobin synthesis. But Levy and his coworkers⁵⁸ made the surprising observation that treatment of Friend cells for 24–30 h is required before the intracellular space is equilibrated with respect to DMSO in the culture medium. Nevertheless, their results with synchronised Friend cells also suggest that the cells do require at least one period of DNA synthesis in the presence of DMSO for haemoglobin to be induced. In contrast, using a Friend cell line of independent origin (T3C12), Leder and Leder⁵⁹ have reported that the inhibition of DNA synthesis with hydroxyurea does not prevent the induction of haemoglobin by butyric acid.

We (P. R. H., D. Conkie, A. Wood, G. Yeoh and J. Paul, unpublished) have attempted to examine this problem at the single cell level, by cloning various Friend cell lines in methocel. In these conditions, most of the cells in clones grown in the presence of various inducers (DMSO, *N*-methylpyrrolidone, *N*-methylacetamide or butyric acid) produce haemoglobin within 3–4 d. Clones arrested at the single-cell stage by the action of hydroxyurea or cytosine arabinoside, or by omission of isoleucine from the culture medium, however, fail to produce haemoglobin; whereas many of the doublet colonies (or binucleate single cells) in the same cultures do produce haemoglobin. Moreover, single cells arrested for 3 d in inducer in the absence of isoleucine do proliferate and produce haemoglobin 4 d after addition of isoleucine. Thus, it seems quite clear that induction of haemoglobin synthesis in our Friend cell lines (which were obtained from Dr C. Friend as were those of McClintock and Papaconstantinou and Levy *et al.*) are dependent on some process within the cell cycle, but that this does not necessarily involve asymmetric division since in many of the doublet colonies both daughter cells contain haemoglobin. Our studies with the T3C12 cell line seem to suggest, in agreement with the findings of Leder and Leder⁵⁹, that a considerable proportion of butyric acid-treated cells arrested with hydroxyurea can produce haemoglobin. Interestingly, in our experiments many of these haemoglobin-containing single cells are in fact binucleate. Thus the discrepancies between the various groups of workers may be more apparent than real, if some process before, or associated with, nuclear division is required for differentiation. Alternatively, butyric acid may act differently from the other inducers; it is also possible that the T3C12 cell line may have been transformed by Friend virus at a later stage of development. Resolution of this question is clearly of importance in elucidating whether, and at what stage, a critical cell division is involved in erythroid cell differentiation.

Friend cell lines are also a useful system in which to attempt more sophisticated molecular and genetic studies of erythroid differentiation. In our own laboratory, a three-stage strategy has been adopted. The first is to obtain stable non-inducible variants; second, these variants are then screened to find out at what molecular level erythroid differentiation is affected; and third, variants are subjected to genetic analysis by cell fusion. By exploiting the fact that the inducible cells differentiate to a non-dividing stage in the presence of DMSO, we can isolate non-inducible variants by continuous growth or cloning in DMSO⁶⁰. Most of our DMSO-resistant lines are also non-inducible by other inducers, although one DMSO-resistant line does respond to *N*-methylpyrrolidone. One DMSO-resistant variant (FtI^{D-}) fails to accumulate globin-specifying sequences in the nucleus, either because of a defect in transcription of the globin genes or instability of the transcripts⁶¹. Moreover,

this defect acts in a *trans*-dominant manner in preventing haemoglobin or globin RNA accumulation in cell hybrids constructed between the FtI^{D-} variant and the normal inducible parent. A second variant, FwI^{D-}, also fails to accumulate haemoglobin⁶⁰ or globin messenger RNAs⁶¹ in response to DMSO or butyric acid, unless it is also treated with haemin⁶¹. This effect of haemin is specific for the FwI^{D-} variant, and cannot be replaced by a precursor to haem (δ -aminolaevulinic acid). Moreover, this defect in inducibility also operates in a *trans*-dominant manner, although loss of a few, as yet unidentified, chromosomes can lead to re-expression of haemoglobin synthesis^{61,62}. Extinction of induction of globin messenger RNA has also been reported by Orkin *et al.*⁶³ in hybrids constructed between high and low inducible cell lines and by Deisseroth *et al.*⁶⁴ in cell hybrids between Friend cells and fibroblasts. Interestingly, however, hybrids between Friend cells and human bone marrow or Chinese hamster erythroid cells could be induced to synthesise globins of both parental types, although in these intraspecific hybrids, extensive segregation of human or Chinese hamster chromosomes had occurred⁶⁵. Thus the general picture which emerges from these studies is that when a Friend cell is fused to a non-inducible, or non-erythroid, cell, extinction of induction of haemoglobin production occurs. This result is similar to that obtained with other differentiating systems (reviewed in refs 66 and 67). Often it has been assumed that extinction of differentiation is due to a negative control system analogous to the bacterial repression systems acting at transcription. In none of these cell fusion experiments cited, however, has it yet been proven that extinction acts at the transcriptional level. For example, although in our own studies we have demonstrated that extinction of haemoglobin synthesis in certain cell hybrids acts at the nuclear level, it has not yet proved possible to demonstrate that globin gene transcripts are not synthesised.

That regulation of differentiation can operate at different molecular levels is illustrated by our molecular analysis of a hybrid cell formed between the Friend cell and the lymphoma cell. This hybrid cell shows extinction of induction of haemoglobin and morphological differentiation, although globin messenger RNA is inducible⁶¹. Both functions are, however, expressed on treatment with haemin in addition to DMSO⁶¹. The precise role of haem in this process requires clarification, for example, by studies of factors in Friend cells analogous to the haemin-controlled repressor in reticulocytes⁶⁸. Furthermore, it is not yet known whether this phenomenon is a property merely of this lymphoma cell line, of lymphoma cells in general or of normal lymphoid cells. But this interesting observation illustrates at the molecular level the diversity of control mechanisms which affect regulation or erythroid differentiation and shows how complex interactions may exist between independent erythroid functions.

Perspectives

The experiments described in this article give some indication of the possibilities of normal erythroid cells and particularly the Friend cell system as a model of a differentiating cell, in which to attempt to dissect the regulatory mechanisms by a combination of cellular, molecular and genetic approaches. It may, therefore, be useful to take a sober look at some of the limitations of the system. The Friend cell is a transformed cell and its relevance as a model for normal differentiation must, therefore, be evaluated critically: as mentioned above, Friend cells seem to be abnormal in that their proliferation is no longer regulated by erythropoietin as in the normal erythroid precursor cell; but differentiation, once it is induced, seems to be relatively normal. Second, the Friend cell is derived from cells transformed after commitment to the erythroid line and the events induced by DMSO may, therefore, be rele-

vant only to the later processes of erythroid differentiation. The important question of the molecular nature of the events concerned with commitment of haematopoietic stem cells to the erythroid line probably cannot be answered with the Friend cell system. Hope that this may prove possible in the future stems from the work of Dexter and colleagues⁶⁹, who have been able to maintain multi-potential stem cells in culture using an appropriate feeder layer. Another promising approach for the future lies in differentiation of teratocarcinoma cells (reviewed in ref. 70) which various groups of workers have recently shown to behave like normal germ cells when transplanted into the environment of the normal embryo at the blastocyst stage of development^{71,72}. It may prove possible to mimic commitment of stem cells to the erythroid line with normal haematopoietic stem cells or teratocarcinoma cells cultured in the appropriate environment. Application to these systems of the kind of molecular/genetic analysis developed for the Friend cell system could then increase enormously our understanding of the relationship between commitment to the erythroid line and subsequent differentiation.

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articles

Accretion flows in the magnetospheres of Vela X-1, AO535 + 26 and Her X-1

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We suggest a model for the complex pulse shapes of the long period, pulsating X-ray sources, Vela X-1 and AO535 + 26. We discuss the flow of accreting matter on to these slow rotators and contrast it with that expected in systems such as Her X-1 which contain a rapidly rotating neutron star.

THE recent discovery of the pulsating X-ray source AO535 + 26 (ref. 1) and unexpected detection of X-ray pulsations in the relatively well studied binary source Vela X-1 (ref. 2) have aroused considerable interest, in part because their pulse periods (104 s in AO535 + 26 and 283 s in Vela X-1) and very complex 1–10 keV pulse shapes (refs 2, 3) are strikingly different

from those of the previously identified pulsating X-ray sources Her X-1 and Cen X-3, which are believed to be rotating neutron stars (see refs 4 and 5).

Several pieces of evidence indicate that in spite of these differences, Vela X-1 and AO535+26 may also be rotating neutron stars. Optical and X-ray observations yield a probable lower bound of $1.4M_{\odot}$ on the mass of Vela X-1 (ref. 6, and references therein), very close to the upper mass limit for slowly rotating white dwarfs. If Vela X-1 is at a distance $D \sim 1.3$ kpc (refs 6, 7), it has a typical X-ray luminosity $L_x \sim 0.2-2 \times 10^{36}$ erg s $^{-1}$ (ref. 2). The distance to AO535+26 is as yet unknown, but its 1975 May high state intensity was comparable with that of the Crab Nebula³, implying $L_x \gtrsim 3 \times 10^{36}$ erg s $^{-1}$ if $D \gtrsim 1$ kpc. Such high luminosities are incompatible with current models of X-ray emission by degenerate dwarfs (see ref. 8). The long rotation periods of these sources may be the result of spin down by a combination of magnetic torques and interaction with surrounding plasma⁹, or the result of spin down in the pre-supernova star, leading to formation of a few neutron stars with spin periods as long as $10-10^3$ s (ref. 10).

Our purpose is, first, to suggest that the complex pulse shapes of Vela X-1 and AO535+26 in the energy range 1–10 keV can be understood if both are slowly rotating neutron stars in which broad, smooth emission pulse shapes, like those seen at 15–30 keV, are modified as the result of cyclotron absorption by clumps of matter entrained in magnetic field irregularities near the stellar surface.

Second, we suggest that accretion flows in the magnetospheres of rapidly rotating neutron stars, such as Her X-1 and Cen X-3, are different from those in slow rotators, and that the observed properties of these sources reflect this difference. In slow rotators accreting plasma probably enters the magnetosphere as a consequence of the Rayleigh–Taylor instability of the magnetospheric boundary (ref. 11), and may plunge close to the surface of the star before being channelled towards polar emission regions by the stellar magnetic field. In fast rotators, on the other hand, the near cancellation of gravitational and centrifugal forces at the magnetospheric boundary may lead to accumulation of matter there, and to more effective channelling of plasma towards the polar caps while it is still far from the star.

We discuss the observational implications of this model of accretion flows, and draw attention to ways in which the differences between fast and slow rotators can be explored observationally.

Accretion by slow rotators

Consider first accretion by slowly rotating neutron stars. Let us assume for the moment that accreting matter approaches a star of mass M approximately radially; at a radius r_s from the star it is shock heated to a temperature $T_s \sim T_{\text{ff}}(r_s)$, where

$$T_{\text{ff}}(r) = GMm_p/k_B r = 1.6 \times 10^{10} (M/M_{\odot}) (r/10^8 \text{ cm})^{-1} \text{ K} \quad (1)$$

in terms of the proton mass, m_p . The radius r_s at which shock heating occurs is determined by the radius, r_A , of the Alfvén surface, where the Alfvén velocity v_A in the stellar magnetic field equals the plasma inflow velocity, v , and the magnetic field of the star first dominates the flow¹². Regardless of the complexity of the magnetic field near the surface of the star, at large distances the stellar field will be dominated by its dipole component; for spherically-symmetric, radial free-fall towards a star with dipole moment μ_{30} , in units of 10^{30} gauss cm³, r_A has the characteristic value

$$r_A^0 = 2.9 \times 10^8 \mu_{30}^{4/7} (M/M_{\odot})^{1/7} L_{37}^{-2/7} R_6^{-2/7} \text{ cm} \quad (2)$$

Here L_{37} is the accretion luminosity in units of 10^{37} erg s $^{-1}$ and R_6 is the stellar radius in units of 10^6 cm. The effect of the star's rotation on the accretion flow can, to a first approximation, be neglected if the angular velocity of the star, Ω_s , satisfies $\Omega_s \ll \Omega_k(r_A) = (GM/r_A^3)^{1/2}$ or, equivalently, $r_A \ll r_c \equiv (GM/\Omega_s^2)^{1/3}$ (ref. 12). This is the condition for 'slow rotation'.

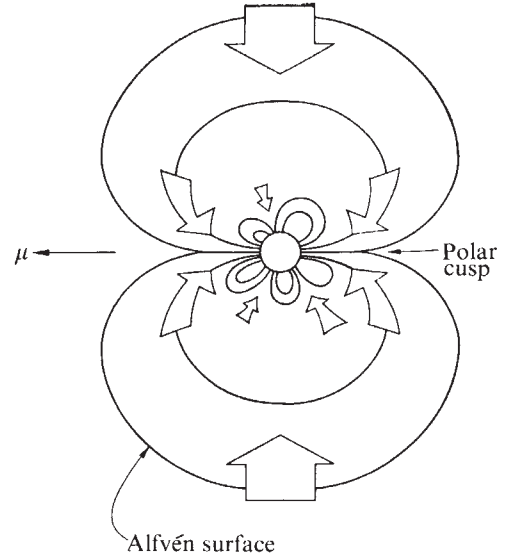


Fig. 1 Suggested plasma flow pattern in slow rotators with complex surface magnetic fields. Plasma enters the magnetosphere as the result of the Rayleigh–Taylor instability of the magnetospheric boundary and plunges close to the stellar surface before being channelled by the stellar magnetic field. Most of the plasma is channelled towards the polar caps, but some flows along field lines that lead to other parts of the stellar surface.

The flow of highly conducting plasma towards the star tends to close the magnetosphere (ref. 12). Since the magnetospheric boundary is convex towards the accreting plasma, the gradient of the field is such as to stabilise the boundary. Nevertheless, the weight of the plasma will drive the boundary locally Rayleigh–Taylor unstable if

$$g_{\text{eff}} = \cos\theta GM/r_m^2 - \kappa B_m^2/4\pi\rho_m > 0 \quad (3)$$

Here θ is the angle between the radius vector and the outward normal to the boundary, r_m is the exact radius of the magnetospheric boundary ($\sim r_A$), B_m and ρ_m are the stellar magnetic field and plasma density at the boundary, $\kappa \sim 1/r_m$ is the curvature of the boundary, and we have neglected radiation pressure forces. If the boundary is in magnetohydrostatic equilibrium, one has $B_m^2/8\pi = n_m k_B T_m$, where n_m and T_m are the particle density and temperature in the plasma just outside the boundary. Thus, the condition for the onset of the Rayleigh–Taylor instability can be written

$$T_m < T_{\text{crit}} = \cos\theta T_{\text{ff}}(r_m)/(2\kappa r_m)$$

where B_m , r_m , and κ are roughly independent of T_m .

Detailed model calculations show that in the absence of cooling, the weight of the plasma is insufficient to drive the boundary unstable (Elsner and Lamb, to be published; see also ref. 13). These calculations show further that instability sets in when the plasma at the boundary has cooled to a temperature $T_{\text{crit}} \sim 0.3T_{\text{ff}}(r_m)$, and that $T_{\text{crit}}/T_{\text{ff}}(r_m)$ is largest at the magnetic equator. Once instability occurs, plasma may penetrate the magnetosphere over much of the magnetospheric boundary except at the axes of the polar cusps, where the boundary is stable. Unfortunately, there are at present no reliable calculations of the properties of the fully developed Rayleigh–Taylor instability in the nonlinear regime, and the precise boundary condition to be imposed on the flow at r_m is therefore unknown. Therefore we adopt the simplest boundary condition that gives a qualitatively correct flow solution: since the growth of the Rayleigh–Taylor instability is rapid and other modes of plasma entry are relatively ineffective, we shall assume that, in a steady state, the region of shock-heated plasma extends a distance Δ_s outside r_m such that T_m is less than but of order T_{crit} .

If the neutron star accretes plasma from a disk, the flow may still resemble in certain respects the flow just described. Thus if the disk thickens near r_A and the flow becomes roughly

Table 1 Characteristics of fast and slow rotators

Source property	Slow rotators ($r_A \ll r_c$)	Fast rotators ($r_A \approx r_c$)
Period changes	Either spin up or spin down, but not both, unless disk flow reverses	Spin down as well as spin up, even if disk flow does not reverse
t_s	$\sim t_s^0$ for disk inflow	$\gg t_s^0$ even for disk inflow
Spectrum	Intense soft (~ 0.1 keV) component not expected	Intense soft (~ 0.1 keV) component possible
Pulse shapes	More complex at lower energies, simpler at higher energies	Simpler at all energies

spherically symmetric there, the flow outside the magnetosphere should resemble the post-shock flow described above. Even if the disk remains thin, the Rayleigh–Taylor instability may well play an important role in allowing the relatively cold disk plasma to penetrate the magnetosphere.

Of particular relevance for the present discussion, recent research on two-dimensional models of accretion disks around magnetic stars (ref. 14 and Ghosh, Lamb, and Pethick, in preparation) suggests that radial flow velocities $v_r \sim v_{ff}$ in the neighbourhood of r_A are a general feature of disk accretion by slow rotators (if shear stresses are large, v_r may become $\sim v_{ff}$ outside the magnetospheric boundary; in any case one expects $v_r \sim v_{ff}$ inside the magnetosphere). If this is the case, one expects $r_A \sim r_A^0$ even for disk inflow¹² and, for disks rotating in the same direction as the star, a spin up timescale, t_s , of order

$$t_s^0 = \frac{I\Omega_s}{(MGM r_A^0)^{1/2}} \quad (4)$$

where I is the star's moment of inertia and M is the mass accretion rate.

For sources with luminosities $L \gtrsim 10^{36}$ erg s⁻¹, the optical depth to Compton scattering of shock-heated plasma outside r_A is

$$\tau_c \approx 0.03 \left(\frac{r_A}{10^8 \text{ cm}} \right)^{-4/5} \left(\frac{M}{M_\odot} \right)^{1/5} \left(\frac{\ln \Lambda}{15} \right)^{-2/5} R_6^{3/5} \quad (5)$$

where $\ln \Lambda$ is the Coulomb logarithm, ~ 15 at r_A . Thus, in slow rotators the rapid development of the Rayleigh–Taylor instability and the large inflow velocities at r_A imply that there is little plasma there to absorb and reradiate keV X rays produced at the stellar surface. An intense soft component ($\varepsilon \sim 0.1$ keV) is therefore not expected in the spectrum of slow rotators.

Pulse shapes

Although the Rayleigh–Taylor instability causes plasma to penetrate the magnetosphere 'between' stellar field lines, the plasma is unlikely to be effectively channelled along these field lines until it is threaded by them; this can occur either by diffusion of the infalling plasma on to stellar field lines or by reconnection of magnetic field lines embedded in the infalling plasma to stellar field lines. If cooling is rapid and threading occurs relatively slowly, the plasma may fall to within a few stellar radii of the star's surface before being funnelled towards the magnetic poles. If in addition the topology of the stellar surface field is complex, with numerous flux concentrations at points other than the poles of the dipolar component of the field, the flow pattern of plasma near the stellar surface may be similar to that shown schematically in Fig. 1.

How long can small scale magnetic flux concentrations be expected to survive in the crust of a neutron star? If the crustal conductivity is σ , such concentrations will decay away on a time scale

$$t_d \approx 4\pi\sigma l_m^2/c^2 = 4 \times 10^6 (l_m/10^5 \text{ cm})^2 (\sigma/10^{24} \text{ s}^{-1}) \text{ yr} \quad (6)$$

assuming they go to a depth $\sim l_m$. To be present now, they may have had to survive for an evolutionary timescale $t_{ev} \sim 10^6$ yr (ref. 15), which would require $\sigma \gtrsim 3 \times 10^{23} \text{ s}^{-1}$ if $l_m \sim 10^5$ cm. Now the temperature of the stellar surface must be $\lesssim 10^6$ K, since otherwise it would be seen as an intense source of soft X rays, and the temperature in the crust itself may be similar. Re-

cent calculations of crustal conductivities¹⁶ show that at this temperature, field decay in the crust from electron–phonon interactions would be entirely negligible after 10^6 yr, while that from electron–charge disorder interactions would be negligible except in lighter stars ($M \lesssim 0.5 M_\odot$) whose crusts are as impure as possible. Decay from scattering off dislocations can also be neglected unless the distance between dislocations is microscopic. Thus, it is entirely reasonable that a large proportion of neutron star X-ray sources may have complex surface magnetic fields.

Since the dipole component of the field is dominant except within a few stellar radii of the surface, most of the matter will still be channelled to the polar caps, as in purely dipolar models, and most of the keV X-ray emission will come from hot spots there, producing broad, smooth pulses at energies $\varepsilon \gtrsim \hbar\omega_c \approx 10 B_{12}$ keV, where B_{12} is the surface field in units of 10^{12} gauss and $\omega_c = eB/m_e c$ is the electron cyclotron frequency. A small fraction of the accreting plasma is, however, likely to be channelled along field lines that lead to other parts of the stellar surface. Then, as the star turns, the broad emission pattern from the polar hot spots will be seen through varying amounts of cyclotron-absorbing plasma, producing complex waveforms at energies $\varepsilon \sim \hbar\omega_c$.

For surface field strengths $B \sim 10^{11}$ – 10^{12} gauss and temperatures $k_B T \sim 10^7$ – 10^8 K one has $\hbar\omega_c \approx k_B T$, and the quantum-mechanical expression¹⁷ for the cyclotron cross section must be used. The optical depth through a clump of dimension size l_c at $\varepsilon = \hbar\omega_c$ is

$$\tau_{cyc} = \frac{l_c \omega_{pe}^2}{c \omega_c} \left(\frac{\pi m_e c^2}{2k_B T} \right)^{1/2} \frac{1}{1 - \exp(-\hbar\omega_c/k_B T)} \quad (7)$$

where ω_{pe} is the electron plasma frequency. Thus one has $\tau_{cyc} \gtrsim 1$ for column densities $\rho l_c \gtrsim 2.9 \times 10^{-5} B_{12} T_8^{1/2} \text{ g cm}^{-2}$, where $T_8 = T/10^8$ K. For clumps of dimension $l_c \sim 10^5$ cm, this represents a density only $\sim 10^{-3}$ that above the polar caps.

In the absence of absorption processes, cyclotron scattering, in which an electron makes a transition from one Landau orbital to the next higher one and back again, is likely to dominate, and will create an anisotropic angular distribution of the radiation emitted by the polar caps without significantly altering its energy distribution. For example, if there are N clumps of size $l_c \sim 10^5$ cm at a distance $d \sim 10^6$ cm from a polar cap, then when a clump is in the line of sight, the X-ray flux at energy ε from that polar cap will be reduced to a fraction $f \sim N(l_c/d)^2 \sim 0.01 N$ of its value when the line of sight lies between clumps, assuming $\exp(-\tau_{cyc}(\varepsilon)) \ll f \ll 1$. On the other hand, if the energy falling on the clumps were thermalised and then reradiated, the temperature T_c of the clumps would be a factor $\sim (l_p/d)^{1/2} \sim 0.3$ lower than that of polar caps of dimension l_p , and the energy distribution as well as the angular distribution would be altered. In either case the spectrum would appear heavily 'absorbed' at low points in the pulse waveform.

Vela X-1 and AO535+26

If AO535+26 and Vela X-1 are magnetic neutron stars with spin periods, P , of 104 and 283 s, respectively, then both are probably slow rotators. Using the estimate (2) for r_A^0 , the condition $r_A^0 \ll r_c$ becomes $L \gg L_{crit}$, where

$$L_{crit} = 1.0 \times 10^{38} P^{-7/3} \mu_{30}^2 (M/M_\odot)^{-2/3} R_6^{-1} \text{ erg s}^{-1} \quad (8)$$

a condition which is easily satisfied by Vela X-1, and by

AO535+26 if it has an accretion luminosity $L \gtrsim 10^{35}$ erg s⁻¹, assuming $\mu_{30} \sim 1$. (Note, however, that L_{crit} depends sensitively on μ .)

Consider now some of the observational implications of the hypothesis that these sources are slowly rotating neutron stars:

(1) If there is a Keplerian accretion disk around the magnetosphere and the disk rotates in the same direction as the star, the star should spin up on a timescale $\sim t_s^0$, with $\dot{P}$ proportional to L . Changes from spin up to spin down should not occur as the result of accretion torques unless the flow departs from this pattern. In the absence of disk flow, t_s should be $\gg t_s^0$, while a reversed disk could lead to spin down on the timescale t_s^0 . (Were these sources degenerate dwarfs, the timescale t_s^0 would be very much longer, since I would be $\sim 10^6$ times larger.)

(2) Because little plasma accumulates near r_A , intense soft X-ray emission is not expected.

(3) If the complex 1–10-keV pulse shapes arise from scattering or absorption by clumps of matter entrained in complex magnetic fields near the stellar surface, as we have suggested, the number and positions of the basic features in time-averaged pulse waveforms should remain fixed, since the field configuration is fixed, although the sizes of various features may change if the flow pattern of the plasma approaching r_A changes. (Were the features instead caused by random clumping of the infalling plasma, independent of the surface field geometry, they should average out on timescales of minutes. Such complicated pulse shapes also seem unlikely to be the result of complex variations in $\dot{M}$ controlled at the magnetospheric boundary¹² since it is there, at $r \gg R$, that the stellar field should be most symmetric; moreover, in slow rotators plasma is likely to enter the magnetosphere over a substantial fraction of its surface, at least in the absence of strong X-ray beaming effects.)

We note that the narrowness of some of the features (in AO535+26, for example, some have widths ≤ 0.05 in pulse phase) favours an interpretation in terms of scattering or absorption rather than beaming, since such narrow features are unlikely to be produced by beaming in quasi-thermal sources such as these, but can easily be produced by absorption if the absorbing clumps are small, close to the stellar surface, and have sizes comparable with those of the polar cap emitting regions.

(4) If the features at 1–10 keV are caused by cyclotron scattering in plasma clumps a distance $\sim d$ above the surface and d is $\sim 10^5$ cm, then the surface fields in both stars must be $\sim 10^{12}$ – 10^{13} gauss. Above 15 keV the polarisation should change smoothly across the pulses (see ref. 19); below 10 keV the polarisation should change smoothly across a given peak in the waveform, but could change abruptly from one peak to the next, since it is the result of absorption of smoothly varying polarised emission at the polar caps by clumps of matter whose absorption polarisations may differ significantly from one to another.

Comparison with fast rotators

We have seen that many of the properties of Vela X-1 and AO535+26 can be understood if they are slowly rotating neutron stars. For comparison, let us consider briefly how the properties of fast rotators, in which $r_A \simeq r_c$, might be expected to differ from those of slow rotators.

If a fast rotator is fed by an accretion disk around its rotation equator, rotating in the same sense as the star, the force of gravity at the disk-magnetospheric boundary will be largely cancelled by centrifugal forces; as a result the growth rate of the Rayleigh-Taylor instability will be reduced and the plasma will fall inward between stellar magnetic field lines more slowly. Thus there will be more time for the accreting plasma to become threaded by stellar field lines far from the stellar surface, perhaps helped along by the Kelvin-Helmholtz instability (see also ref. 13), and channelling of the flow by the stellar field

is therefore likely to occur at $r \gg R$. Finally, since the dipolar component of the field dominates at $r \gg R$, the flow is likely to be more effectively funnelled towards the two polar caps than in slow rotators.

If this difference in the accretion flow does occur, it will be reflected by the properties of such sources:

(1) If an extreme fast rotator is surrounded by a Keplerian accretion disk rotating in the same direction as the star, the accretion torque caused by material stresses at r_A can be largely cancelled or even reversed by magnetic and viscous stresses. Thus, even if such a source is disk fed, the average spin up timescale should be longer than t_s^0 , and occasional episodes of spin down would be expected.

(2) If the infall of plasma is significantly slowed by near cancellation of gravity by centrifugal forces, a substantial quantity of matter may accumulate near r_A , producing a soft X-ray component in the spectrum by reprocessing keV X rays emitted at the stellar surface⁵. If sufficient matter accumulates, the pulsed fraction in the energy range 0.1–1 keV will be reduced, and the pulse phase in general shifted from that at 1–10 keV.

(3) If the slowly infalling plasma is effectively channelled along stellar field lines far from the star's surface, essentially all of the plasma may be funnelled towards the two polar caps, producing broad, smooth pulses throughout the keV energy range.

These expected properties of fast rotators surrounded by accretion disks are well illustrated by Her X-1, which has a spin period of 1.24 s. Assuming $M = 1.3M_\odot$ (ref. 20), $R_0 = 1.5$ (ref. 21), and $L_{37} = 5.8$, appropriate to a distance of 6 kpc (ref. 7), equation (2) gives $r_A^0 \sim r_c$ for $\mu_{30} \sim 1$, indicating that Her X-1 is indeed a fast rotator. Even though Her X-1 is believed to be surrounded by a Keplerian accretion disk⁵ it shows episodes of spin down as well as spin up⁴. Moreover, the observed average spin up time, 3×10^5 yr (ref. 22), is a factor ~ 40 longer than the slow-rotator estimate (4), if $I \simeq 2 \times 10^{45}$ g cm² (ref. 21). This factor is too large to be accounted for by uncertainties in I or in r_A (since $t_s^0 \propto r_A^{-1/2}$, r_A would have to be a factor $\sim 10^3$ smaller than our estimate), and is strong evidence that material stresses at r_A are closely cancelled by magnetic and viscous stresses. In addition, there is an intense soft X-ray component in the spectrum of Her X-1 (ref. 23) that has been interpreted in terms of a centrifugally-supported opaque shell of accumulated matter partially surrounding the neutron star magnetosphere (ref. 24; reprocessing by plasma at the magnetospheric boundary has also been mentioned in ref. 25). Such an accumulation of matter cannot be supported by magnetic forces or radiation pressure since it would be Rayleigh-Taylor unstable on a dynamical timescale. Finally, the pulse waveforms seen in Her X-1 are comparatively simple throughout the energy range 1–30 keV, as would be expected if all the accreting plasma were funnelled towards the two polar caps. (Alternatively, these simple pulse shapes could arise simply from the absence of complex surface magnetic fields in Her X-1, but we believe this possibility is less likely.)

If a fast rotator is fed by more nearly radial inflow, or by a disk which is not aligned with the stellar rotation, the accretion flow is likely to be more complicated with a possibility of centrifugally-supported matter in the rotation equator and rapid inflow near the rotation axis.

Discussion

We have suggested that Vela X-1 and AO535+26 are slowly rotating neutron stars in which complex 1–10-keV pulse shapes are produced by cyclotron absorption near the stellar surface, and have indicated several ways in which this hypothesis can be checked.

We have also suggested that accretion flows in the magnetospheres of fast rotators are different from those in slow rotators, and that the properties of X-ray sources will reflect

this difference. Some of these properties are summarised in Table 1. Further study of other pulsating X-ray sources will show whether this model of accretion flows is correct. Thus SMC X-1, with a recently discovered period of 0.71 s (ref. 26) and a luminosity $\sim 6 \times 10^{38}$ erg s $^{-1}$ is a prime candidate for fast rotation, while 3U1728-24, with a period of 123 s (refs 27, 28) and a luminosity (if it lies near the galactic centre) $\sim 10^{38}$ erg s $^{-1}$, should be a slow rotator. It is also possible that 3U1813-14, 3U1223-62, and 3U0352+30, which have recently been reported to be pulsating²⁷, are slowly rotating neutron stars.

A further implication of this model of accretion flows is that a rapidly rotating neutron star located in a binary system where the mass transfer rate is very high might be completely surrounded by a centrifugally-supported opaque cloud. If such a cloud smears out periodic intensity variations without reprocessing too large a fraction of the keV X-ray emission into soft X rays, it could account for the observed properties of unpulsed sources like Sco X-1 and Cyg X-2 (ref. 5); such a possibility has also been recently suggested by Sunyaev (private communication through R. McCray).

We conclude by pointing out that a given source can change from fast rotation to slow rotation, or vice versa, if it is 'choked' or 'starved' of accreting matter. One source that might show such transitions is Cen X-3, which shows some characteristics of both types of accretion flows and apparently undergoes large changes in accretion rate²⁹. Although Cen X-3 usually has a comparatively simple pulse shape and also shows occasional spin down episodes³⁰, both of which are characteristics of fast rotators, equation (2) gives r_A only $\sim 0.3 r_c$ for $P = 4.84$ s, $\mu_{30} \sim 1$, and $L_{37} \sim 6$ (appropriate for an X-ray high state at a distance ~ 10 kpc), and the average spin up timescale is $\sim 3 \times 10^3$ yr, in rough agreement with the slow rotator result, equation (4). Cen X-3 has been observed to have a more pronounced double pulse structure during what is believed to have been a 'choke-out' (Fabbiano and Schreier, personal communication), but the observation appears inconclusive as a test of the accretion flow pattern. The enhanced pulse-to-pulse variations

in pulse shape seen during one 'starve-out' are probably unrelated to the increased complexity in the time-averaged waveform expected were Cen X-3 to become a true slow rotator. Clearly, observation of a transition from fast to slow rotation would provide an important check on the model.

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Membrane action of DMSO and other chemical inducers of Friend leukaemic cell differentiation

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DMSO and other cryoprotective agents produce a pronounced increase on the phase transition temperature of phospholipid membranes, indicating an increased stability. The effects of DMSO and the other cryoprotective agents, divalent cations, and local anaesthetics on the transition temperature of phospholipid membranes seem to correlate with their effects on the differentiation of Friend leukaemic cells in vitro. These studies suggest that the induction of differentiation by cryoprotective agents may be the result of the interaction of these agents with cell membranes.

GROWTH of Friend leukaemic cells (FLC) in suspension culture in the presence of any one of a variety of cryoprotective agents results in the differentiation of these cells along the erythroid pathway^{1,2}. Although evidence has been

presented that DMSO must ultimately influence the control of differentiation at the level of transcription³, the primary site of action of these agents remains unknown. The known properties of these agents as penetrant-carriers and cryoprotectants suggested to us that their initial and perhaps major effects might be on the cell membrane. Considerable speculation has recently been directed towards possible alteration of membrane properties during cell differentiation and malignant transformation⁴. We have used differential scanning calorimetry (DSC) to study the effects of these agents on membrane properties. This technique has been used extensively to obtain a direct measure of a temperature and enthalpy of the lipid phase transition from the solid to the liquid-crystalline state^{5,6}. The temperature at which the phase transition occurs in a given system is related to the fluidity of the membranes, and lipid fluidity has been shown to be an important parameter in various functions of biological membranes⁷⁻⁹.

The studies reported here demonstrate that DMSO and

other compounds which induce cell differentiation, alter the properties of phospholipid membranes causing the appearance of a new transition at higher temperatures. This effect indicates stabilisation of the membranes and we interpret it as evidence for decreased membrane fluidity. Local anaesthetics, which increase membrane fluidity^{10,11} inhibit the artificial membrane effects as well as the induction of benzidine positivity in FLC *in vitro* by these chemical agents. Divalent cations enhance the effects of the inducing agents and reverse the inhibitory effects of the local anaesthetics on the artificial membranes and on FLC growing in suspension culture. These studies suggest that interaction of chemical inducing agents with membrane phospholipids may be involved indirectly in the induction of FLC differentiation by the same agents.

Effects of DMSO on phospholipid membrane transition temperature

Our initial studies were with DMSO, the first inducer recognised and one of the most potent agents available. Figure 1 demonstrates the effect of increasing concentrations of DMSO on the thermotropic properties of DMPG. Increasing concentrations of DMSO produce a shift of the T_c of the normal transition towards higher temperatures (mid-point T_c from 33.0 to 36.8 °C), while apparently reducing its magnitude until it disappears (curve *e*). DMSO also causes the appearance of a previously unrecognised endothermic transition at much higher temperatures (60–70 °C) which also shifts towards higher temperatures with

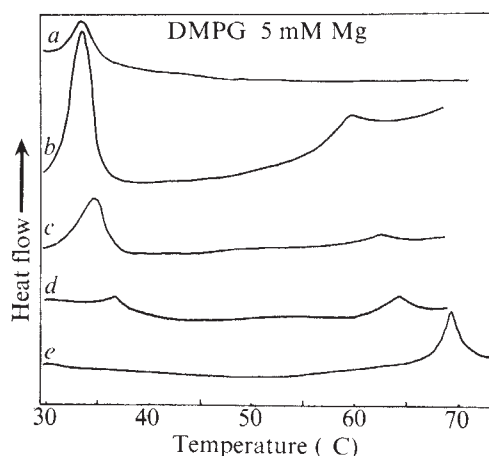


Fig. 1 The phospholipid used in these studies was dimyristoyl-phosphatidylglycerol (DMPG) synthesised as described previously¹². It was stored under nitrogen in sealed ampoules at –50 °C at a concentration of approximately 10 $\mu\text{mol ml}^{-1}$. Multilamellar vesicles were prepared by the method of Bangham *et al.*¹³. The phospholipid was dispersed in phosphate-buffered saline (PBS) prepared in the presence of 5 mM magnesium chloride but free of calcium. Following dispersion at 37 °C, 2–3 μmol of lipid were distributed into individual sample tubes to which was added 5–10 ml of the solution to be studied. The vesicles were then incubated in PBS in the presence of 5 mM magnesium chloride for 3 h at 37 °C in the following conditions: *a*, controls, no additions or in the presence of; *b*, DMSO 2% (v/v), 0.26 M; *c*, DMSO 10% (v/v), 1.3 M; *d*, DMSO 20% (v/v), 2.6 M; *e*, DMSO 30% (v/v) 3.9 M. After incubation, the lipid suspension was centrifuged at 2,000 r.p.m. for 30 min and the wet pellet transferred to a sample pan and covered. Using a PBS standard, the phase transition temperature (T_c) of the phospholipid vesicle suspension was determined using a Differential Scanning Calorimeter (DSC-2, Perkin-Elmer, Norwalk, Connecticut). In all the studies a sensitivity scale of 1 mcalorie s^{-1} , a chart speed of 20 mm min^{-1} and a scanning rate of 50 °C min^{-1} were used. The T_c , defined as the midpoint of the transition temperature, was found by heating the lipid samples at the indicated rate. The amount of lipid in each sample was not identical in each sample, so that the total heat of the transition which is represented by the area under the peak varies with different samples. Consequently, comparisons between different samples were made only with respect to the T_c , and with the relative area under each peak when two peaks were present.

Table 1 Effects of chemical inducers

Inducer	Benzidine-positive cells* (%)			
	Experiment No. 1		Experiment No. 2	
Control§†	0.3	0.3	0.6	0
DMSO 1% (0.13M)	8	14	4	9
DMSO + Mg†	24	19	37	—
DMSO + Ca†	17	17	18	19
DMSO + Dib.†	3	8	0	1
DMSO + Dib. + Ca†	11	13	9	5
TMU (4.3 × 10 ⁻³ M)	38	38	47	47
TMU + Proc.†	26	14	24	13
TMU + Proc. + Ca†	45	47	42	34

*Friend leukaemic cells (FLC), line 745A, were grown in suspension culture in Eagle's basal media with Earle's balanced salts (Gibco), made 15% with foetal calf serum (FCS) (Gibco). The cells were incubated at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% room air. Cultures were seeded at 1×10^5 cells ml^{-1} and routinely passed at 3–4-d intervals. The chemical agents under study were added either alone or in combination at the time of seeding of the culture into 2-ml plates in duplicate. After 5 d of growth the cultures were collected, counted, and slides were made using a centrifuge, stained with benzidine Wright–Giemsa and the proportion of benzidine-positive cells was determined by one observer who counted 300 cells in a single blinded fashion. Cell viability was determined by Trypan blue exclusion.

†Concentrations: Mg^{2+} , 8×10^{-3} M; Ca^{2+} , 14.3×10^{-3} M; Dibucaine, 5×10^{-6} M; Procaine, 7.3×10^{-4} M. Procaine hydrochloride was from McGraw Laboratories, Glendale, California and magnesium chloride, $6\text{H}_2\text{O}$ from Fischer Sci. Co., Fairlawn, New Jersey.

‡Control cultures had no agents added. The benzidine-positive cell counts for Mg, Ca, Dibucaine, and Procaine control cultures were identical to the control counts noted above.

§Standard BME contains 8×10^{-4} M Mg^{2+} and 1.9×10^{-3} M Ca^{2+} . For the cases where additions were made the final concentrations were as indicated†.

increasing concentrations (Fig. 1). The upper transition first appears at a DMSO concentration of 2% (v/v), it becomes predominant at about 20% (v/v) and is the only transition present at 30% (v/v). It should be noted that the optimal concentration of DMSO for inducing FLC differentiation is 2% (v/v) which is in the range of observed effects on phospholipid membranes seen here. The appearance of the new transition at high temperature was associated with visible flocculation of the lipid preparation following dispersion of the sample.

Effects of other inducers of differentiation on transition temperature

As shown earlier^{1,2} DMSO, DMF, PNO, and TMU are all effective inducers of erythroid differentiation of FLC *in vitro*. The results presented in Fig. 2 indicate that all these agents induced the appearance of a new transition at higher temperatures (upper T_c) which is similar to that produced by DMSO. The temperature of the upper transition did not always rise with increasing concentrations as observed with DMSO. The effects of the compounds on the lower peak varied somewhat with some compounds causing an increase (curve *b*) in the lower T_c with increasing concentration, while others caused little change (curves *c* and *d*) or a small decrease of the lower T_c (curve *e*). Butyric acid (data not shown) is unique among the effective inducers studied¹⁴ in that at low concentrations (0.01 M) it raises the T_c while at higher concentrations (0.1 M) it lowers the T_c , and at even higher concentrations (1.0 M) it eliminates the transition in the temperature range tested. It was not established whether butyric acid produced a new transition at much higher temperatures.

The urea family of compounds has been found to have varying effects on the induction of erythroid differentiation in FLC in the following order: TMU is an extremely potent inducer of differentiation, DMU is a moderate inducer and urea a very weak inducer². At concentrations of 30% (v/v) in the absence of Mg^{2+} , urea had no effect on the lower T_c and did not produce a new transition at higher temperatures.

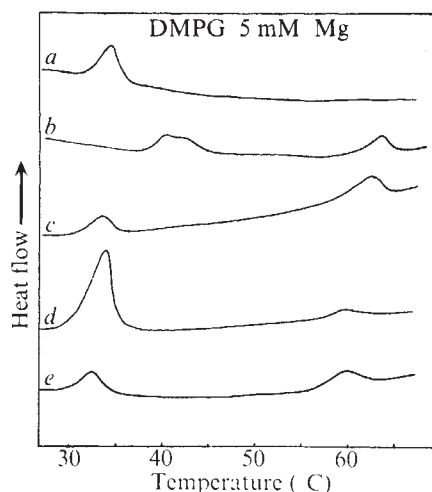


Fig. 2 Differential scanning calorimetry of DMPG vesicles in the presence of various inducers of erythroid differentiation. Vesicles were prepared in the presence of PBS with 5 mM magnesium chloride: *a*, Control. All further calorimetry thermograms were obtained in the same conditions except for the presence of: *b*, DMSO 15% (v/v), 1.95 M; *c*, DMF 15% (v/v), 1.95 M; *d*, PNO 10% stock solution (v/v), 0.81 M; *e*, TMU 10% (v/v), 0.86 M. PNO stock solution was 8.1 M, made by dissolving 100 g PNO in 130 ml of sterile water. The agents used in these studies were used as provided or dissolved in PBS (1 l contains: 900 ml 0.145 M NaCl; 80.40 ml 0.067 M Na_2HPO_4 ; 19.60 ml 0.067 M KH_2PO_4) and were obtained from the following companies: dimethylsulphoxide (DMSO), pyridine-*N*-oxide (PNO), tetramethylurea (TMU), dimethylurea (DMU), and butyric acid (BA), all from Eastman Kodak, Rochester, New York; dimethylformamide (DMF), urea, Fischer Sci. Co., Fairlawn, New Jersey.

On the other hand, both DMU and TMU were effective in abolishing the lower transition and producing one at much higher temperatures. TMU, which is the most potent inducer of differentiation in our system², in the presence of 5 mM Mg^{2+} caused the appearance of an upper transition at the lowest concentration tested (0.2% v/v), which is clearly within the range for inducing differentiation of FLC *in vitro* (0.1% v/v is optimal concentration).

Antagonistic effects of local anaesthetics and chemical inducers

It has previously been demonstrated that local anaesthetics increased the fluidity of acidic phospholipid membranes as indicated by a decrease of the T_c (ref. 11). Since the inducing agents were found to have the opposite effect on the T_c , we studied the interactions of these agents in the same system (Fig. 3).

As discussed above, the presence of DMSO results in an upward shift of the normal T_c and the appearance of a new transition of much higher T_c (Fig. 3, curve *c*). Dibucaine, on the other hand, lowers the normal T_c (curve *d*). When the vesicles were exposed simultaneously to dibucaine and DMSO, the upper T_c disappeared and the lower T_c was at midpoint between the peaks obtained when each agent was present alone (curve *e*). The disappearance of the upper T_c coincided with the disappearance of the DMSO-induced flocculation associated with the appearance of the upper T_c .

The addition of calcium to the mixture of DMSO and dibucaine not only restored the DMSO-induced upper peak, but also diminished the size of the lower transition compared with that shown by DMSO alone at that concentration (curve *f*). The exothermic (inverted) peak shown in curve *f* at 26°C is usually obtained at higher concentrations of DMSO alone (30%). A more detailed study of the synergism of the effects of DMSO and divalent metals will be reported elsewhere²².

Divalent cations and local anaesthetics on differentiation induction

The effects of inducing agents, divalent cations, and local anaesthetics on the properties of artificial phospholipid membranes led up to examine their effects on FLC growing in suspension culture. Friend leukaemic cells were grown in the presence of each of these agents alone or in combination (Table 1). DMSO 1% (v/v) induces a low degree of erythroid differentiation. Increased concentration of calcium and, to a somewhat greater extent magnesium, seem to enhance the ability of DMSO to induce differentiation although they do not induce differentiation on their own at the concentrations used. Both dibucaine and procaine were found to inhibit to a large extent the development of benzidine positivity induced by DMSO and TMU in terms of both the proportion of benzidine-positive cells in the culture as well as the degree of positivity of the individual cells. Furthermore, calcium was able to restore and perhaps slightly enhance the DMSO-induced benzidine positivity previously inhibited by the local anaesthetics. None of these compounds alone or in combination, nor the addition of calcium, had any effect on the number or viability of cells present after 5 d of culture when compared with control cultures. Two additional observations are of interest. When FLC were grown in higher concentrations of DMSO or TMU (at 0.27 M or 8.6×10^{-3} M respectively) the topical anaesthetics produced little if any inhibition of benzidine positivity. Additionally, although the addition of calcium had no effect on the rate of cell growth, the benzidine-positive cells in these cultures were vacuolated.

Membrane effects of cryoprotective agents

Cryoprotective agents which induce Friend leukaemic cell differentiation *in vitro* have variable effects on the normal T_c of phospholipid vesicles but consistently induce the appearance of a new endothermic peak at higher temperature. We interpret this as evidence for a stabilisation of the lipid bilayers indicating a decrease in membrane fluidity. Although high concentrations of inducing agents were frequently used, detectable effects on membrane fluidity were obtained with concentrations in the range used to induce differentiation in culture. Our previous studies with cryoprotective agents suggested that the ability of an agent to induce FLC differentiation paralleled the basicity of the agent¹. Our current studies are in accord with these in that we have found that the effects of these agents in 'stabilising' phospholipid membranes are much more pronounced with acidic than with neutral phospholipids²² it therefore seems that ionic charge interactions and perhaps hydrogen bonding¹⁵ may be involved in the interaction of these compounds with phospholipid membranes.

The DSC studies demonstrated that agents, such as local anaesthetics^{10,11,16} which increase membrane fluidity, inhibit the DMSO effect on membranes, whereas on the other hand, factors which decrease membrane fluidity such as Ca^{2+} and Mg^{2+} ^{17,18}, enhance the DMSO effect. An analogous relationship was found between the actions of divalent metals (enhancing) and a local anaesthetic (inhibiting) on cell differentiation induced by DMSO as judged by the development of benzidine positivity. But the inhibition of benzidine positivity by local anaesthetics¹⁹ raises several questions in itself, since not only was the proportion of benzidine-positive cells decreased, but the degree of benzidine positivity of the differentiating cells was also reduced. This latter observation raises the possibility that the local anaesthetics may interfere only with the synthesis of haem and perhaps not inhibit at the same site where DMSO is involved directly in inducing FLC differentiation. For example, DMSO and other inducing agents have several different effects, whereas the local anaesthetics may merely inhibit mitochondrial synthesis of

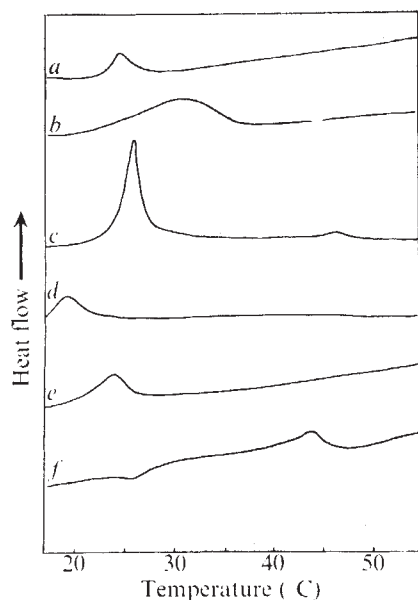


Fig. 3 Differential scanning calorimetry of DMPG vesicles in the presence of DMSO, dibucaine and calcium. *a*, Thermogram obtained from vesicles prepared in the presence of PBS alone. The remaining curves were obtained from vesicles to which were added: *b*, calcium chloride 5×10^{-4} M; *c*, DMSO 20% (v/v), 2.6 M; *d*, dibucaine hydrochloride 5×10^{-5} M; *e*, DMSO 20% (v/v), 2.6 M and dibucaine hydrochloride 5×10^{-5} M; *f*, DMSO 20% (v/v), 2.6 M, dibucaine hydrochloride 5×10^{-5} M, and calcium chloride 5×10^{-4} M. Dibucaine hydrochloride was obtained from K and D Laboratories, Plainview, New York.; calcium chloride from Upjohn Co., Kalamazoo, Michigan.

haem. Other manifestations of differentiation may therefore not be inhibited by the local anaesthetics.

Our studies suggest that the cryoprotective ability of these compounds may also be related to their membrane effects. It is possible that a decrease in fluidity of the cell membrane could render the cell and its subcellular organelles more resistant to distortion during freezing. In fact, preliminary experiments in our laboratory suggest that local anaesthetics may inhibit DMSO cryoprotection of erythrocytes.

Although not ruling out a direct effect on transcription, the studies reported here suggest that in FLC line 745A interaction of the cryoprotective inducers with the cell

membrane may play an important part in the induction of differentiation of murine erythroleukaemic cells. A decrease in membrane fluidity could alter the transport and permeability properties of the membrane or cause the 'release' of an intracellular messenger which would itself be responsible for initiating the transcription of those mRNAs necessary for erythroid differentiation. On the other hand, the same physical interactions which occur between the agents and the phospholipid membranes and the associated water molecules could also occur at other sites within the cell (including proteins, or nucleic acids). The conformational changes produced at such sites^{20,21} could result in differentiation by directly effecting the transcription of mRNAs associated with differentiation. Although these lipid vesicle studies seem to be predictive of the effects of cryoprotective agents on FLC line 745A, they are not predictive of their effects on line 745D, a cell line which is refractory to DMSO and only minimally responsive to other cryoprotective agents². It thus seems possible that the difference in responsiveness between lines 745A and 745D may be a result of differences in their cell membranes.

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Similarity of the crystal and solution structure of yeast tRNA^{Phe}

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A comparison of the crystal and the solution structure of yeast tRNA^{Phe} has been carried out by calculating the low field NMR spectrum from refined X-ray structure co-ordinates. The similarity between the computed and observed spectra show that the crystal and solution structure are virtually identical.

NUCLEAR magnetic resonance (NMR), in theory, can provide information on the solution structure of a macromolecule at the same level of resolution as obtained with X rays in solids. In particular, the chemical shift and relaxation parameters contained in an NMR spectrum are sensitive functions of the local environments of individual nuclei. Nevertheless, the transition from the theoretical argument to the practical task of attempting to extract such information from NMR spectra poses significant problems since the magnitude of individual effects are not precisely known and difficult to calculate.

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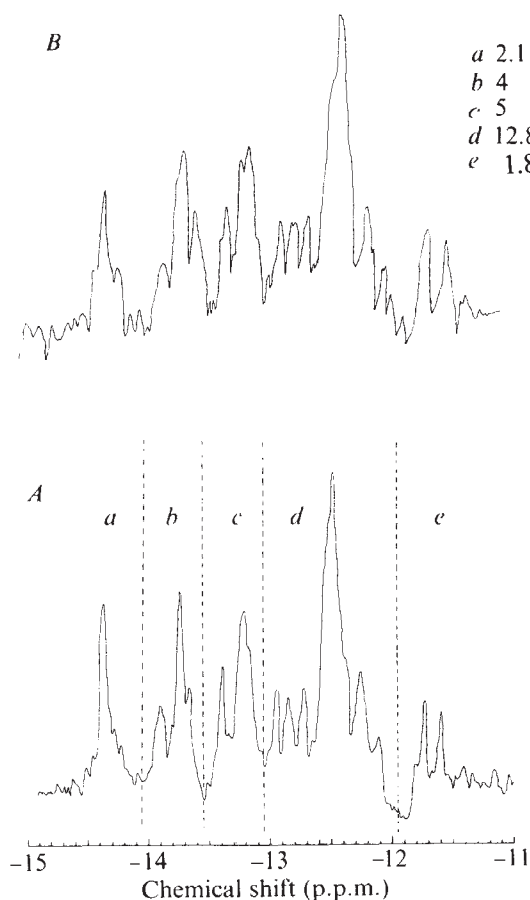
Table 1 Optimised ring current values

Nucleotide	Ring currents	
	Giessner-Prettre and Pullman ⁶	Optimised
Adenine		
Hexagonal ring	0.88	0.763
Pentagonal ring	0.67	0.580
Guanine		
Hexagonal ring	0.25	0.288
Pentagonal ring	0.63	0.725
Cytosine	0.27	0.210
Uracil	0.08	0.107

Calculation of magnetic ring currents, however, has reached a significantly high level of accuracy to be useful in predicting resonance positions if these positions, to a reasonable approximation, are determined only by ring current effects. In the case of NMR spectra of tRNA it is likely that ring currents are the dominant shift mechanism determining resonance positions.

Each ring NH...N hydrogen bonded proton in a Watson-Crick base pair generates a resonance in the low field region of the proton NMR spectrum of tRNA¹. The chemical shift of a proton in such an environment is a combination of three principal effects: (1) deshielding by polarisation of the NH bond due to the adjacent electronegative atoms of the hydrogen bond; (2) deshielding arising from the ring currents of the two aromatic rings between which this hydrogen bonded proton is located; and (3) shifts caused by the ring currents of bases

Fig. 1 360-MHz proton NMR spectra of yeast tRNA^{Phe} in H₂O solutions taken at 35 °C. The spectra represent 1-h accumulations in the continuous wave mode with a sweep rate of 12 s per 2,400 Hz. Solvent composition was: A, 1 mM tRNA, 1 mM EDTA and 10 mM sodium cacodylate pH 7; B, 1 mM tRNA, 1 mM EDTA, 10 mM sodium cacodylate pH 7, 15 mM MgCl₂, 0.1 M NaCl.



stacked above and below the base pair containing the proton in question. The magnitude of these three effects may be as large as several parts per million. All other shift mechanisms generate shifts on the order of a few tenths of a part per million.

Since the X-ray structure of yeast tRNA^{Phe} has now been solved^{2,3} and refined coordinates are available, we have calculated, directly from the coordinates, the ring current shift which each hydrogen bonded proton experiences from all other aromatic rings in the molecule. The results show that, on the basis of the resulting NMR spectrum, the X-ray crystal structure and the solution structure are virtually identical.

Ring current calculations

We have used the theory of Haigh and Mallion⁴ to calculate the ring current shifts of the hydrogen bonded protons. In this theory, which is based on McWeeny's quantum mechanical treatment⁵, all contributions to the ring current shifts are standard geometric terms that are calculated from the positions of ring atoms, multiplied by a factor proportional to the magnitude of the ring current. Haigh and Mallion used an empirical factor that is relevant only for in-plane protons attached to aromatic ring systems, but that should be scaled up by a factor of 2.6 for large distances. We have used this factor of 2.6 as appropriate for the distances occurring in tRNA. Our program for calculating the ring current shifts places a proton on a straight line between the donor and acceptor nitrogens at a distance of 1.02 Å from the donor. It then sums the ring current contributions of every ring in the molecule excluding the donor and acceptor rings for the base pair of interest. These rings are considered separately when determining starting positions for a hydrogen bonded proton in a given type of base pair.

Ring currents for the four isolated bases A, G, U and C have been calculated by Giessner-Prettre and Pullman⁶ and are expressed relative to the ring currents in benzene (see Table 1). For several reasons calculated values of ring currents can be considered to be only approximately correct: (1) ring currents, derived from second-order perturbation theory, are sensitive to approximations used in the quantum mechanical treatment; (2) in hydrogen bonded base pairs the ring currents may deviate substantially from those in isolated bases; (3) the neglect of the effect of charges in heterocyclic rings; and (4) the inaccuracy of the geometric terms used in the calculation of shifts all may influence the apparent ring currents. Empirical adjustments for each of the ring currents should therefore be made to properly account for the observed shifts. We used the calculated ring currents⁶ as starting values in an iterative procedure that successively refined each value. First, the A and G ring currents, and the difference between the AU and GC starting positions were varied and the computer was allowed to make a best fit between the nearest members of the calculated and the observed spectra. Then the C and U ring currents were refined keeping A and G fixed. These new values then provided the basis for a final slight refinement of A and G ring currents. In each case, the pentagonal and hexagonal ring currents of each purine were varied by the same factor, thus leaving only four ring currents to be refined. Because of the rapid convergence of this technique, no further iteration was required. A major advantage of this procedure is that one is not required to make detailed *a priori* assignments of given base pairs to specific resonances in the observed spectrum and thereby prejudice the outcome.

The values arrived at by this optimisation procedure are listed in Table 1. As can be seen, the optimum values for this calculation are only slightly different from the values calculated by Giessner-Prettre and Pullman⁶. Furthermore, the changes are consistent with those expected from hydrogen bonding between base pairs. During hydrogen bonding the electron density in the electron cloud, and hence the ring current, is expected to increase on the donor base and decrease on the acceptor base. This is precisely what occurs when the ring

Table 2 Base pair ring current shift

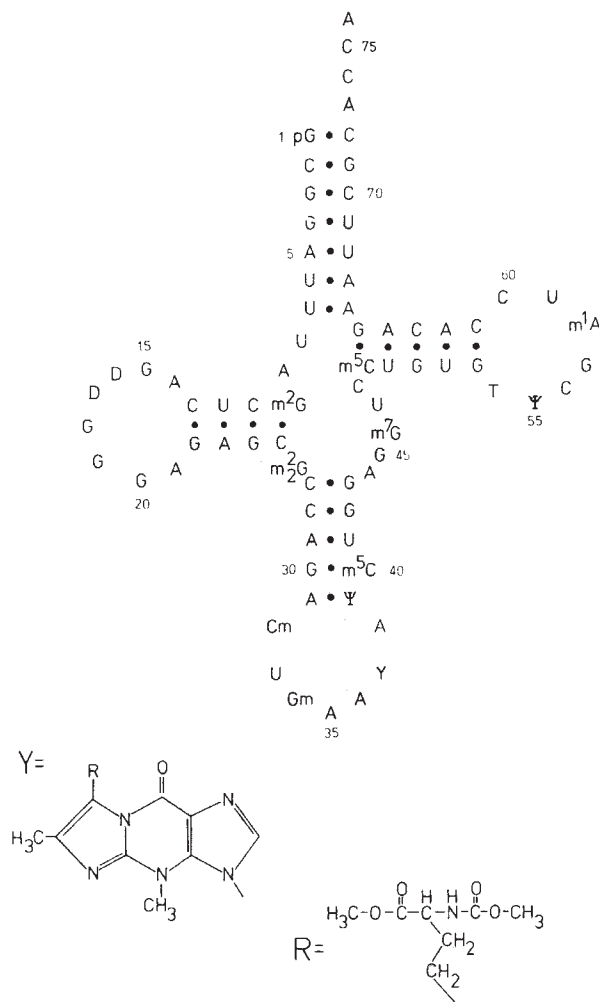
Helix	Base pair	Ring current shift (p.p.m.)
Acceptor	GC 1	+1.2244
	CG 2	+1.0705
	GC 3	+1.4032
	AU 5	+0.6150
	UA 6	+0.6151
	UA 7	+1.0451
DHU	GC 10	+0.9852
	CG 11	+0.4379
	UA 12	+0.3418
	CG 13	+0.5539
Anticodon	CG 27	+1.1602
	CG 28	+1.9580
	AU 29	+1.1530
	GC 30	+0.9366
	AΨ 31	+0.2392
TUC	CG 49	+1.3464
	UA 50	+1.5445
	GC 51	+1.2773
	UA 52	+1.1299
	GC 53	+0.9840
Tertiary	U8-A14	+0.5369
	T54-A58	+0.4942
	G22-m ⁷ G46	+1.7710
	G19-C56	+0.8615
	m ³ G26-A44	+0.4052
	G15-C48	+0.4003

currents are optimised. We are confident, therefore, that the optimisation procedure has not led to incorrect answers.

Yeast tRNA^{Phe} NMR spectrum

For the purpose of making detailed comparisons between observed and calculated spectra, it was necessary to use the most highly resolved spectra of tRNA^{Phe} available. Figure 1 shows the low field NMR spectra of yeast tRNA^{Phe} taken at 360 MHz in the absence or presence of Mg²⁺ (B. R. Reid, personal communication). Except for slight shifts in resonance positions and the narrowing of the resonances in the absence of Mg²⁺, the two spectra are identical. Because of the higher magnetic fields and the special solvent conditions, however, the

resolution in the no Mg²⁺ spectrum (Fig. 1A) reveals a number of features not observed in previously published spectra of this molecule⁷⁻¹⁰. There are seven well resolved resonances below -11.5 p.p.m. all of which have approximately unit intensity. When the total integrated intensity in the spectrum is calculated relative to any one of these individual resonances we find 26 ± 1 protons between -15 and -11.5 p.p.m. as shown in the table on the side of spectrum in Fig. 1B (B. R. Reid and G. T. R., in preparation). Similar spectral resolution plus an independent calibration on the high field methyl resonances has led us recently to the same conclusion in the case of *Escherichia coli* tRNA_I^{Val} (refs 11 and 12). The intensity in the low field region of yeast tRNA^{Phe} must then be attributed to the 20 secondary structure Watson-Crick hydrogen bonded base pair protons

Fig. 2 Cloverleaf sequence of yeast tRNA^{Phe} (refs 14 and 15).

(Fig. 2) plus hydrogen bonded protons from tertiary interactions. There are five tertiary interactions involving ring NH...N type hydrogen bonds which should account for five of the resonances in this region. Only one proton resonance is left unaccounted for and its origin will be discussed in the following section.

Figure 3A is a computer fit of the original spectrum in Fig. 1A. It was prepared by assigning a number of Lorentzian lines of equal intensity and linewidths to the positions where they occur in the original spectrum. Twenty-six lines were used to generate this spectrum and comparison with Fig. 1 demonstrates that the integration shown in this figure accurately reflects the number of protons in each peak of the original spectrum. The individual positions used in this fit were the same as those used in the ring current optimisation discussed above. The optimised ring current values for the four bases obtained by this procedure (Table 1) have been used in the Haigh and Mallion ring current

Table 3 Proton resonance positions

Base pair	Chemical shift (p.p.m.)	
	Calculated	Observed
UA 12	-14.01	-13.91
AU 5	-13.74	-13.72
UA 6	-13.74	-13.72
UA 7	-13.31	-13.38
UA 52	-13.22	-13.25
AU 29	-13.20	-13.22
AU 31	-13.18	-13.18
CG 11	-13.10	-13.16
CG 13	-12.98	-12.93
UA 50	-12.81	-12.83
GC 30	-12.60	-12.57
GC 10	-12.55	-12.51
GC 53	-12.55	-12.49
CG 2	-12.47	-12.47
CG 27	-12.38	-12.42
GC 1	-12.31	-12.39
GC 51	-12.26	-12.27
CG 49	-12.19	-12.23
GC 3	-12.13	-12.09
CG 28	-11.58	-11.58
T54-A58	-14.38	-14.40
U8-A14	-14.42	-14.40
G19-C56	-12.68	-12.72
G22-m ⁷ G46	-12.47	-12.47
G15-C48	-11.78	-11.72
m ³ G26-A44	-13.81	-13.81

Base pair offsets: Watson-Crick AU, -14.35; Watson-Crick GC, -13.54; reversed Hoogsteen AU, -14.90; AΨ ≈ -13.50.

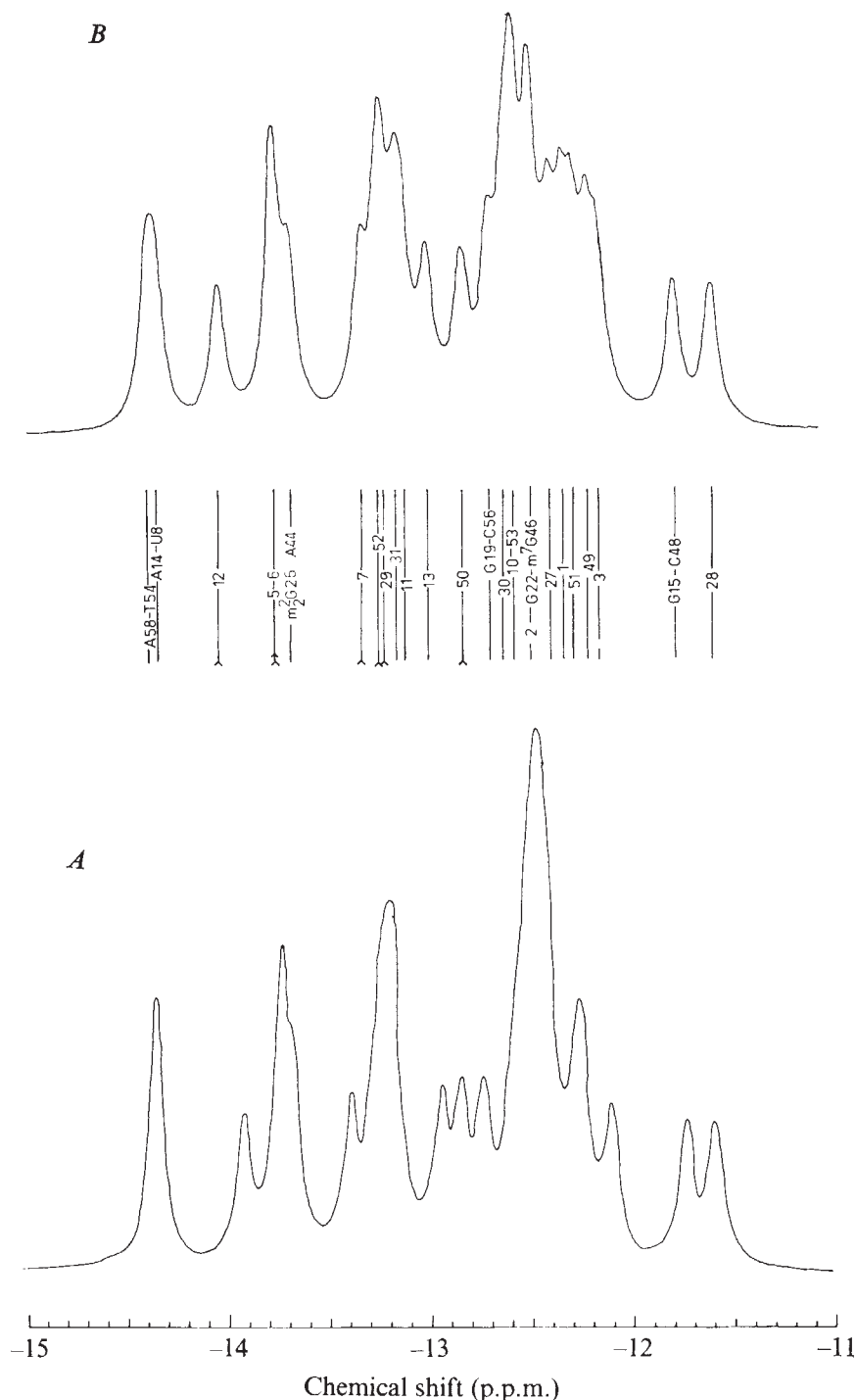


Fig. 3 *A*, Computer fit of the spectrum in Fig. 1*A* prepared as described in the text; *B*, spectrum calculated from the X-ray structure coordinate and optimised ring current values using the procedures and offsets described in the text and Tables 1-3. The indicated base pair positions are those assigned by the computer from the calculations. Secondary structure resonances are denoted by the normal conventions 1, 2 Secondary structure AU resonances are denoted by an $\wedge$ at the bottom of the line. Tertiary structure resonances are denoted by the full lettering and numbering of the base pair, that is, U8-A14 and so on.

calculation along with the X-ray crystal structure coordinates of yeast tRNA^{Phe} refined by Sussman and Kim (personal communication). The resulting spectrum (Fig. 3*B*) compares very closely with the original (Fig. 3*A*). Since the NMR spectrum of tRNA^{Phe} can be quite accurately calculated from the crystal structure coordinates simply by summation of the ring currents arising from each aromatic substituent in the molecule, there must be a very high degree of similarity between the solution and crystal structure.

Resonances from secondary structure base pairs

A base pair hydrogen bonded proton will experience deshielding ring currents from the aromatic rings of the donor and acceptor bases. If the distance between the donor and acceptor bases varies slightly, as they do in the refined crystal structure coordinates (2.8-3.1 Å), there will be slight alterations in the ring

current shift. Since it is not clear, however, that these differences are physically meaningful at the current level of resolution of the X-ray coordinates, they have been ignored in the present calculations. Any error which results from this approximation will be small since the differences in degree of deshielding originating from these slight changes amount to only a few Hz. Deshielding also arises from the electronegative atoms of the hydrogen bond as stated previously. Since it is impossible to determine accurately the hydrogen bond strengths and correlate them with deshielding effects we have again assumed that the deshielding arising from the hydrogen bonded situation of the proton is the same for a given type of base pair. Thus the starting position for a proton in each type of isolated base pair is allowed to be a variable in the optimisation procedure discussed above. The parameters found were -14.35 and -13.54 p.p.m. for Watson-Crick AU and GC base-paired hydrogen bonded protons, respectively.

Resonance from A Ψ base pair

The aromatic ring of the "Y" base is stacked in the anticodon loop, one ring removed from the A Ψ base pair. In the present calculation the ring current shift arising from this base was calculated using the ring current value provided by B. Pullman (personal communication). The total ring current shift experienced by the A Ψ hydrogen bonded proton is 0.24 p.p.m. In studies of the anticodon fragment of yeast tRNA^{Phe}, Lightfoot *et al.*⁹ found the A Ψ resonance occurring in region *c* of Fig. 2 and set the starting position at -13.5 p.p.m. We find that the A Ψ could occur in either region *b* or *c* depending on the starting position and the magnitude of the ring current shift which the proton experiences. In the absence of other information, therefore, we prefer to rely tentatively on the fragment studies and assign the resonance to region *c* with the A Ψ starting position in the region of -13.5 p.p.m.

Resonances from tertiary structure interactions

The calculated resonance positions marked in Fig. 3 show that the two protons in region *a*, and one each in regions *b*, *c*, *d* and *e* are not normal Watson-Crick AU or GC secondary structure resonances. Having assigned A Ψ 31 to region *c* in the previous section, the tertiary interactions must generate resonances in the remaining regions. The tertiary structure interactions involving U8-A14 and T54-A58 are of the reversed Hoogstein type (see Fig. 4*b*). These two tertiaries experience approximately the same ring currents as do the hydrogen bonded protons of AU5, 6 and 12 (see Table 2). If the AU offset is the same for both Watson-Crick and reversed Hoogstein base pairs, five protons fall into region *b* and none into region *a* of Fig. 1. It is probable, however, that protons in the reversed Hoogstein arrangement have a starting position

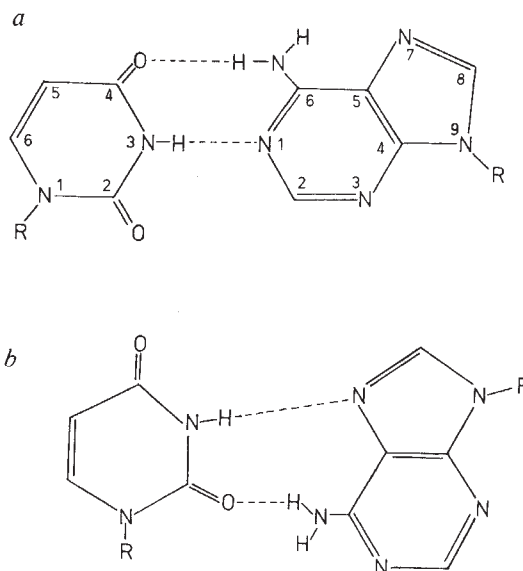


Fig. 4 Comparison of Watson-Crick AU base pairing (a) and reversed Hoogstein AU base pairing (b).

to lower field than Watson-Crick AU protons. In the reversed Hoogstein arrangement (Fig. 4*b*), the hydrogen bond acceptor is N7 of the five-membered ring instead of N1 of the six-membered ring. It has been shown by calculations on the tautomeric forms of purines¹³ that the ring currents in both the hexagonal and pentagonal rings are a factor of 2-3 higher if a proton is attached to the N7 compared with the N1. The change in ring currents on hydrogen bonding N7 in place of N1 is

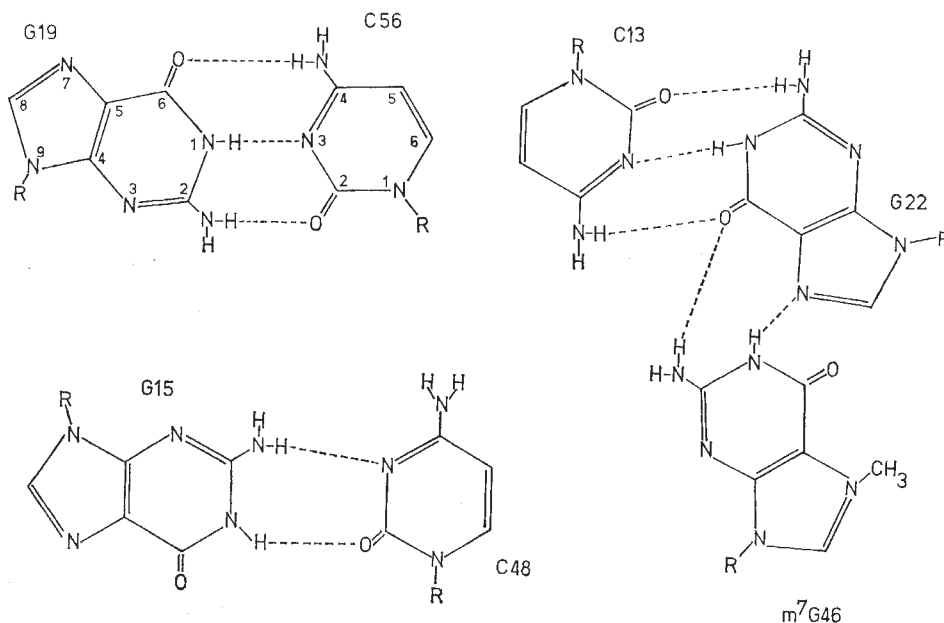


Fig. 5 Tertiary structure base pairing interactions, excluding the AU reversed Hoogstein, which are suggested to contribute resonances in the low field region of the NMR spectrum of yeast tRNA^{Phe} (refs 2 and 3).

expected to be in the same direction. Thus the deshielding experienced by the proton in the reversed Hoogsteen pair is expected to be greater than in the Watson-Crick base pair and the starting position should occur at lower field. Setting the reversed Hoogsteen AU offset at -14.9 p.p.m. places the two tertiaries U8-A14 and T54-A58 in peak *a* at -14.4 p.p.m. A lower field starting position for the reversed Hoogsteen base pair may also arise from a localisation of positive charge on the five-membered ring when hydrogen bonding occurs at N7. In contrast, the positive charge which develops when hydrogen bonding occurs at N1 in a Watson-Crick arrangement may be withdrawn from the ring by resonance interaction with the NH_2 group. A higher degree of positive charge on the ring would result in greater deshielding and a lower field position for the reversed Hoogsteen AU base pair proton.

Figure 5 shows the remaining tertiary structure interactions which are likely to give rise to resonances in the -15 to -11.5 p.p.m. region of the NMR spectrum. Since G19-C56 is a normal Watson-Crick base pair we have used the standard GC starting position of -13.54 p.p.m. which places the resonance from this base pair in region *d* (see Fig. 3). Only one proton each in regions *b*, *d* and *e* remain unaccounted for. Allowing for the strong deshielding ring currents which should arise from both rings in the tertiary base pair $\text{m}_2^2\text{G26-A44}$, the starting position for this resonance should certainly be lower than a normal Watson-Crick GC and may be more nearly the same as a Watson-Crick AU proton resonance. Therefore, we have tentatively placed $\text{m}_2^2\text{G26-A44}$ in peak *b* with a starting position of -14.2 p.p.m. The G22- $\text{m}_2^7\text{G46}$ interaction (Fig. 5), is of the reversed Hoogsteen type. By analogy with the arguments presented for the AU reversed Hoogsteen resonance starting positions we expect this GG reversed Hoogsteen offset to be to lower field than a Watson-Crick GC starting position. The difference between a Watson-Crick and a reversed Hoogsteen AU offset is 0.55 p.p.m. With the G22- $\text{m}_2^7\text{G46}$ resonance assigned to region *d* at -12.47 p.p.m. the GG reversed Hoogsteen interaction has a starting position of -14.2 p.p.m. (0.65 p.p.m. to lower field than the Watson-Crick GC interaction). The ring NH of G15 hydrogen bonded to the exocyclic oxygen of C48 should be less deshielded than a normal Watson-Crick GC and we have tentatively assigned this proton to region *e* as indicated in Fig. 3 with a low field starting position of approximately -12.1 p.p.m.

Comparison with previous NMR data

The original analysis of the yeast tRNA^{Phe} NMR spectrum was made with considerable help from NMR spectra of fragments of the individual helices⁹. Since the helical region structure was expected to be fairly stable in the presence or absence of the rest of the molecule, the spectra obtained were assumed to reflect the resonance positions of the base pairs for a given helix in the intact molecule. If one compares the fragment data with the assignments which have evolved from the present calculations of the secondary structure resonance positions, it is immediately evident that the present assignments are in very close agreement with the fragment data. The only noticeable deviation is the assignment of GC 13 in the DHU helix which was originally assigned to one of the two highest field resonances. Our calculations show, however, that GC 13 suffers very little upfield shift (see Fig. 3 and Table 2).

The major change which must be made as a result of these studies is that the Watson-Crick AU starting position is changed to -14.35 p.p.m. In the early studies, without knowledge of AU tertiary structure resonances it was necessary to set the offset to lower field to account for the resonances which occurred below -14 p.p.m. This, however, necessitated raising the A and G ring currents 20% above published values.

The results of removing the sulphur from thiouridine in *E. coli* tRNA^{Val} (ref. 11) provide further support for assigning the resonances in region *a* of Fig. 1, to the tertiary AU resonances. When the sulphur was removed from s₄U8 by cyano-

gen bromide treatment the resonance which occurred at -14.9 p.p.m. in the NMR spectrum of *E. coli* tRNA^{Val} disappeared and was replaced by a new resonance at -14.2 p.p.m. This treatment should have changed a s₄U8-A14 tertiary base pair into a normal U8-A14 tertiary base pair as we have in yeast tRNA^{Phe}. The new resonance which appeared was located very close to that which we have assigned to the U8-A14 tertiary in the present spectra.

Kallenbach *et al.*¹⁶ observed resonances at -13.8 and -13.2 p.p.m. when studying AMP interacting with oligo(U₁₅). Assuming the Watson-Crick AU offset of -14.6 p.p.m. originally used by Shulman *et al.*⁸, the -13.8 p.p.m. resonance was assigned to ring $\text{NH} \cdots \text{N}$ Watson-Crick interactions while the -13.2 p.p.m. resonance was assigned to hydrogen bonded protons in the reversed Hoogsteen interactions. The offsets proposed in this paper reverse the order of these assignments. Furthermore, when these offsets are used in conjunction with new ring current shift tables (Robillard *et al.*, in preparation) the position of the Watson-Crick and reversed Hoogsteen resonance interactions for this polymer are predicted to occur exactly at the observed positions.

In their studies of ribosyl ApApGpCpUpUp double-stranded helix, Kan *et al.*¹⁷ observed an AU resonance assigned to the internal AU base pair. At 0° the resonance occurred at -14.2 p.p.m. Since this base pair proton is expected to receive an upfield shift of approximately 0.5 p.p.m. from the neighbouring G, it would seem that the present starting position of -14.35 p.p.m. for a Watson-Crick AU base paired proton resonance is incorrect. It should be realised, however, that the temperature-dependent measurements of this fragment show a continuing shift as a function of decreasing temperature even after changes in the resonance line widths have ceased. As has been shown¹⁷, this could arise from a structural change in the helix geometry (fraying effects). Thus it is likely that these fragments studies are not directly comparable with studies on larger polymers.

Conclusions

Table 3 contains the resonance positions of the protons in the observed spectrum and compares them with those which we have calculated using the offsets given at the bottom of the table and the ring current shifts in Table 2. The r.m.s. error between the resonances in the two columns is less than 0.05 p.p.m. (18 Hz) when only the 20 secondary structure resonances are included. The agreement between the calculated and observed positions, which is even within the observed linewidth, can be considered as very satisfactory since in the optimisation procedure only five adjustable parameters were used to calculate 20 relative line positions. These parameters were the four ring currents and the difference between the AU and GC starting positions. The deviations which are still observed may well represent slight differences between crystal and solution structure. They may also arise from a slightly incorrectly refined structure in one part of the molecule. Further experiments are in progress to examine these possibilities as well as the accuracy of our optimised ring currents and starting positions for the individual base pair types.

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letters to nature

The 21-cm absorption line in NGC1275

DE YOUNG, Roberts and Saslaw¹ detected H I absorption in NGC1275 with a velocity of 8,120 km s⁻¹. This is ~ 3,000 km s⁻¹ larger than the systemic velocity of NGC1275 and is the same as that of the enigmatic system of gas filaments extending 40'' from the nucleus^{2,3}. The observations of De Young *et al.* were made with a single dish with insufficient angular resolution to determine which of the various radio continuum sources were being absorbed. They argue that the absorption is probably occurring in the components < 0.2'' rather than in the 5' halo component, but they were unaware of an intermediate scale 30'' component⁴. Here we present observations made with sufficient angular resolution to decide unambiguously between these possibilities. Our data can also be used to place a limit on the H I mass of possible foreground objects which could be causing the absorption.

Table 1 Flux densities in each channel

$c\Delta\lambda/\lambda_0$ (km s ⁻¹)	Average flux densities† (Jy)		Relative flux densities‡ (Jy)	
	'short' (50'')	'long' (20'')	'short' (50'')	'long' (20'')
8,085	10.86	9.37	-0.039	-0.053
8,126	10.62	9.14	-0.285	-0.291
8,167	10.89	9.41	-0.013	-0.017
8,209	10.91	9.42	-0.016	-0.008
8,250	10.91	9.42	-0.004	-0.006
8,291	10.91	9.43	0.005	0.001
8,332	10.92	9.43	0.011	0.004
8,373	10.90	9.43	-0.011	0.001

* Corrected to heliocentric.

† Calibrated with respect to 3C48 and 3C147 (assumed flux densities 15.91 and 21.90 respectively). The errors in these values are $\pm(0.007+1\%)$.

‡ With respect to the channels with the highest four velocities.

The observations were obtained with the Westerbork Synthesis Radio Telescope and its 80-channel filter spectrometer⁵. The measurements with the ten shorter baselines (36–684 m in 72-m increments) were made on August 11, 1974, and those with the 10 longer baselines (756–1,404 m in 72-m increments) on November 15, 1974. The eight frequency channels have a half-power bandwidth of 129 kHz ($c\Delta\lambda/\lambda_0 = 27$ km s⁻¹) and cover the range in $c\Delta\lambda/\lambda_0$ from 8,085 to 8,373 km s⁻¹. Table 1 gives the amplitudes in each channel averaged at the position of the continuum source. For the shorter baselines this corresponds to the flux density from a region ~ 50'' × 75'' in diameter (EW × NS) centred on the nucleus of NGC1275. For the longer baselines this is the flux density from components < (20'' × 30'') at the position of the nucleus.

For each observation, the variations in amplitude for the highest four velocities are consistent with that expected from receiver noise, so we have taken the average of these four channels to represent the mean continuum amplitude and have also tabulated the amplitudes relative to this value. To compare

our result with that of De Young *et al.*¹, we have convolved their profile with our broader bandpass, and we have used the more accurate gain calibration of the 300' antenna⁶. This gives an absorption of 0.26 ± 0.02 Jy, in agreement with our value of 0.287 ± 0.006 Jy in the 8,085 km s⁻¹ channel.

Table 2 lists the angular sizes and flux densities of the main components of NGC1275 at the epoch of these observations. The deepest absorption seen by De Young *et al.*¹ corresponds to 1.4 Jy, so it is possible that this could have occurred in any of the three continuum components. Figure 1a shows the fringe amplitude of continuum emission in position angles 0° and 90°. The three angular size scales as described by Miley and Perola⁴ are clearly seen. In Fig. 1b we show the difference between the channel containing the strongest absorption and the channels containing the continuum. It is clear from this plot that the absorbed component is unresolved and we can place a limit of < 6'' EW and < 9'' NS on its angular size. Furthermore, from the interferometer phase we can see that the position of the absorbed component is within 1'' of the position of the small diameter continuum source.

From this we can exclude the possibility that the major part of the absorption is occurring in either the 3' × 4' halo component or the 30'' intermediate component but it is still possible

Fig. 1 *a*, The fringe amplitude of the continuum emission from NGC1275 in position angles 0° (×) and 90° (●) plotted against the interferometer baseline in wavelengths. Note that the amplitude scale is offset from zero. *b*, The difference between the channel containing the strongest absorption and the average of the channels containing the continuum emission.

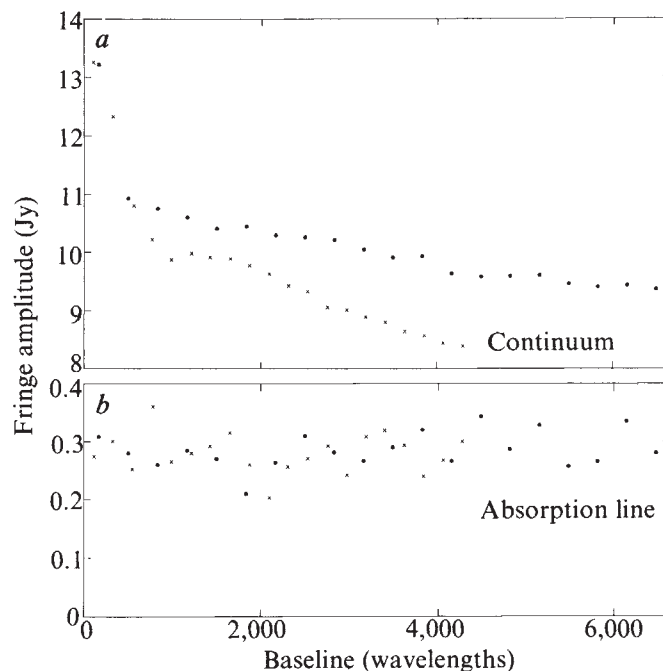


Table 2 Flux densities and absorption of the components

Component	Size*	Flux density (Jy)	Absorption (Jy)	%
Compact	<0.05''	8.6 [†]	0.29	3.4
Intermediate	35'' × 12''	2.2	<0.07	<3§
Halo	4' × 3'	2.0¶	<0.05	<2

* Half intensity diameters of equivalent Gaussian distributions. Major axis position angle 170°.

† Average over our 27-km s⁻¹ bandwidth.

‡ Variable—see text.

§ Upper limits are 3 × r.m.s.

¶ The continuum fringe amplitudes in Fig. 1 also include a contribution from NGC1265 at the shorter spacings.

that a smaller fraction of the total absorption is occurring in these components. We can set limits on this fraction by fitting Gaussian components of the appropriate sizes to the absorption visibility in Fig. 1b. The resulting upper limits which correspond to 3 × r.m.s. are also given in Table 2. Since the limits correspond to a fractional absorption in the larger scale components less than that seen in the compact component we can just exclude an absorbing cloud at 8,126 km s⁻¹ uniformly covering the larger scale components, but we could not exclude a non-uniform cloud.

Table 3 summarises information from Westerbork on the variability of the continuum emission from the compact component at 1.4 GHz. The continuum emission has increased by 15% since 1971 and this is similar to the 10% increase between our present value of the absorption and that seen by De Young *et al.*¹. The different calibration and velocity resolution of these two observations makes this result rather uncertain, however. Further accurate monitoring of the absorption line would indicate whether the continuum component responsible for the absorption is the variable component or some other part of the complex of small scale continuum structures known to be present⁷.

One of the possible hypotheses to explain the absorption is that there is a foreground galaxy or H I cloud moving towards NGC1275 with a velocity of +3,000 km s⁻¹. Such a large positive velocity is not impossible if it is a member of the Perseus cluster, which has an r.m.s. velocity dispersion of 1,500 km s⁻¹. To detect such an object we can either search for emission at velocities away from the absorption line velocity or search for emission from a larger region than that seen in absorption.

Since we do not see any feature in Table 1 exceeding the noise level we can set a 3σ limit of < 0.024 Jy in a 27-km s⁻¹ channel on any emission feature in the velocity range 8,167 to 8,373 km s⁻¹. Comparison of the 50'' and 20'' data enables similar limits to be put on any emission at the absorption velocity coming from an area of ~ 50'' diameter centred on the galaxy. This corresponds to 18 kpc for an assumed distance of 75 Mpc for NGC1275.

For a foreground object smaller than or comparable with this size with a velocity dispersion Δ*V* in km s⁻¹ our flux density limit gives a limit to the mass of neutral hydrogen, *M*_{H I}, of

$$M_{\text{H I}} < 3 \times 10^7 (\Delta V) M_{\odot}$$

If Δ*V* is much greater than 200 km s⁻¹ our limit is less well defined since the total profile would be wider than our velocity coverage. Thus a galaxy having the same distribution of H I

velocities as the optical filaments (Δ*V* ≈ 250 km s⁻¹) would have *M*_{H I} < 7.5 × 10⁹ *M*_⊙. A late-type galaxy with this profile width would have *M*_{total} ≲ 8 × 10¹¹ *M*_⊙ and *M*_{H I} ≈ 6 × 10⁹ *M*_⊙ (ref. 8), which is just below our present detection limit.

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New limit on the size distribution of γ-ray bursts

SINCE the discovery of γ-ray bursts of non-solar origin in 1973 (ref. 1), about 42 events have been confirmed. All have been observed by X-ray and γ-ray detectors on various satellites, though a number of unconfirmed bursts have also been observed by balloon-borne instruments². The lack of confirmation in the latter cases is not surprising, because of short exposure time and differing sensitivities. The detected flux ranges between about 3 × 10⁻⁶ erg cm⁻² for the smallest burst to 5 × 10⁻⁴ erg cm⁻² for the largest observed burst. The integral size distribution of these events above 10⁻⁴ erg cm⁻² follows an *S*^{-1.5} law, suggesting an isotropic distribution of the sources. For events with energy of less than 10⁻⁴ erg cm⁻², however, the observed distribution seems to flatten out, and the choice of number index (α = 0.5, 1.0 or 1.5) in this region is not strongly supported by experimental results.

We present here a new upper limit for the number of bursts which deposit more than about 2 × 10⁻⁷ erg cm⁻² in the energy range 100–1,000 keV. The result indicates the direction in which future γ-ray burst experiments might go.

Our instrument comprised a 125-mm NaI(Tl) detector 10 mm thick, shielded from photons originating in the lower hemisphere by a graded shield. The pulses were analysed in four integral channels between 40 and 1,000 keV. The prescaling factor and the sampling rate for each channel was matched to the available telemetry capacity. The counting rate between 100 and 1,000 keV was sampled every 0.6 s, for 300–1,000 keV and 600–1,000 keV every 2 s, while counts between 40 and 1,000 keV were read out 6 times in 5 min. The instrument was flown as a piggy-back experiment on the first transatlantic balloon flight launched at 0505 UT on August 15, 1975 from Trapani, Sicily (38°N, 13°E). The balloon reached a ceiling altitude of about 3.0 g cm⁻² at 0740 UT, and floated for 78 h before being cut down over Kentucky, USA³. The data were transmitted using a high-frequency radio link and were recorded at two ground stations, one in Sicily and the other in Chesapeake Beach, USA. The region of sky observed during this flight included a complete band across the celestial sphere defined by δ = +38 ± 60°.

The counting rates observed in each energy channel indicated the perfectly normal behaviour of the instrument. The changes in the detector counting rates from time to time were well understood in terms of variations in the residual atmospheric depth and other detector parameters. Since this transatlantic flight was the first of its kind, good scientific data were obtained for only about 70% of the time at float altitude. The loss of data arose mostly from the particular configuration of the high frequency communications system used on this proving flight. The criterion

Table 3 Continuum variability of the compact component at 1.4 GHz

Date	Frequency (MHz)	Flux density (Jy)
June 5, 1971	1,415	7.3 ± 0.1
August 11, 1974	1,383	8.6 ± 0.1
October 10, 1974	1,415	8.7 ± 0.1
November 15, 1974	1,383	8.6 ± 0.1

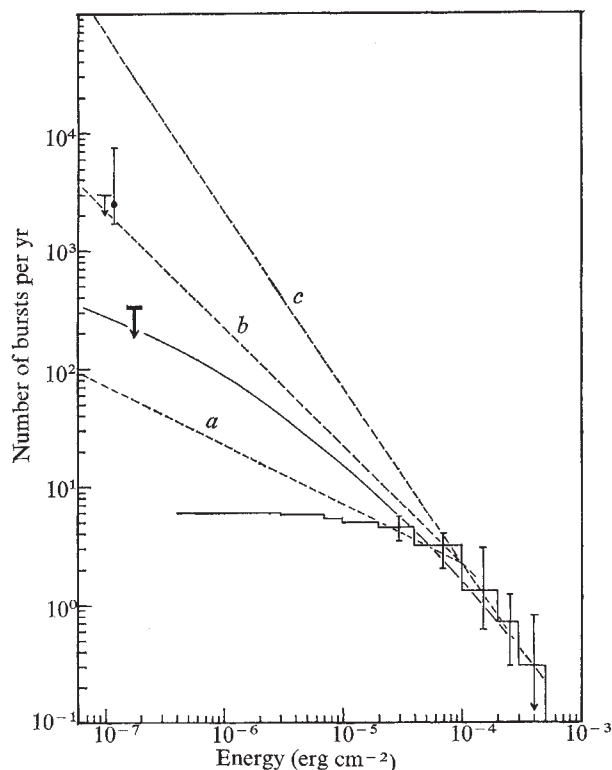


Fig. 1 Observed data of γ -ray bursts along with the results reported here. The limits defined by Cline *et al.* are those presented in ref. 5. Solid line, modified galactic distribution, $S^{-1.0}$ with a break point around 5×10^{-5} erg cm $^{-2}$ and the dotted lines represent the form $N \propto S^{-\alpha}$ where $\alpha = 0.5$ (a), 1 (b) or 1.5 (c) for one-two- and three-dimensional distribution; stepped line, Vela events; ●, ref. 2; thin arrows, ref. 4; thick arrows, our results.

for the detection of a burst was that the counting rates per interval should each increase by more than 3σ above the background rate in at least three successive 0.6-s intervals. No candidate burst event could be found in the data. Such an excess would have corresponded to a signal strength of 0.36 photon cm $^{-2}$ in the energy region 100–1,000 keV. This is equivalent to 0.1 photon cm $^{-2}$ in terms of the Vela detector threshold, assuming an E^{-2} differential photon spectrum. Figure 1 shows the $\log N$ - $\log S$ distribution observed for Vela events along with the upper limit derived from this experiment. It can be seen that the limit is a factor of 5 lower than that expected from an S^{-1} distribution and about 100 times smaller than the value expected from an $S^{-1.5}$ number distribution. A 95% confidence upper limit would be higher than the plotted value by a factor of 3. That limit is still lower than that expected, even on the assumption of a two-dimensional distribution of burst sources. The present results favour the $S^{-0.5}$ spectrum for γ -ray bursts.

The results seem to demonstrate that the bursts are not of extragalactic origin, otherwise they would give an isotropic distribution. Similar conclusions have been suggested by Strong *et al.*³ on the basis of the general features of observed γ -ray bursts. A galactic distribution of nearby sources would follow an $S^{-1.5}$ distribution for sources up to 300 pc, which is typical of the thickness of the galaxy. It would then change to an S^{-1} distribution for sources up to 3 kpc, which is the distance to the galactic anticentre, and then tend towards an $S^{-0.5}$ distribution beyond that point. If the break point at 10^{-4} erg cm $^{-2}$ in the Vela data is genuine, and occurs because of the limited thickness of the galaxy, then one would also expect to find a less defined change of slope at about 10^{-6} erg cm $^{-2}$. On this basis, the expected number of bursts would be about 3 times greater than the upper limit obtained in our detector. But a pure disk population of burst sources with an S^{-1} distribution, and with a Gaussian distribution in its intrinsic source luminosities, would have a steeper slope on the $\log N$ - $\log S$ curve for energies greater than that expected from the mean luminosity. A $\log N$ -

$\log S$ distribution has been calculated for such a disk population with a mean luminosity corresponding to a detected energy of 10^{-4} erg cm $^{-2}$. This also includes a further flattening between 10^{-5} and 10^{-6} erg cm $^{-2}$ which arises when sources are at a distance of 3 kpc.

We believe that our results are consistent with a size distribution of $S^{-1.5}$ above 10^{-4} erg cm $^{-2}$ and $S^{-0.5}$ below this energy, as determined for the Vela bursts. A distribution function obtained from a galactic population with a Gaussian spread in intrinsic luminosities having a break point at $\sim 5 \times 10^{-5}$ erg cm $^{-2}$ would, however, also be compatible with our data. The break point at 10^{-4} erg cm $^{-2}$ in the case of the Vela events may arise because of the characteristics of the Vela detection system, since it is optimised for events having a fast rise time. A slowly rising burst could escape detection. For example, the event of April 27, 1972 was seen by Apollo 16 but was not detected by the Vela satellites.

In view of the two competing spectra, $S^{-0.5}$ and S^{-1} , modified by a Gaussian distribution in luminosities, a reduction by a factor of about 10 in the limit obtained by this experiment is needed to establish the true nature of the source origin. Clearly, reducing the threshold sensitivity much below that of the present detector has little advantage, whereas the extension of the observation period reduces the limit in direct proportion to the flight length. Exposure times of up to 500 h could be obtained in relatively few flights using the techniques developed for the transatlantic balloon facility. It is important that some of the future long duration flights should be made in the Southern Hemisphere, where the galactic centre would pass within the field of view of the detectors. When quasi-omnidirectional detectors are used, it will be necessary to qualify any result on the size spectrum of γ -ray bursts with information on the sky coordinates of the region observed. The true form of the size spectrum is of extreme importance in the development of theories to explain γ -ray bursts. Some attempts at a theoretical explanation of this class of event have been made, but any quantitative picture will change drastically if the size spectrum follows either of these two distribution or, in the extreme case, if the distribution has a cutoff at lower energies.

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Palynomorphs indicating Permian rocks in Ethiopia

KARROO (Carboniferous to Triassic) strata have been reported in Ethiopia^{1,11,12}, but without firm documentation. Other sediments lying on the crystalline basement but beneath the Jurassic Adigrat Sandstone² may also be of Karroo age³⁻⁵. To our knowledge, however, the occurrences reported here are the first palaeontologically substantiated records of Permian strata in Ethiopia. Localities are shown in Fig. 1.

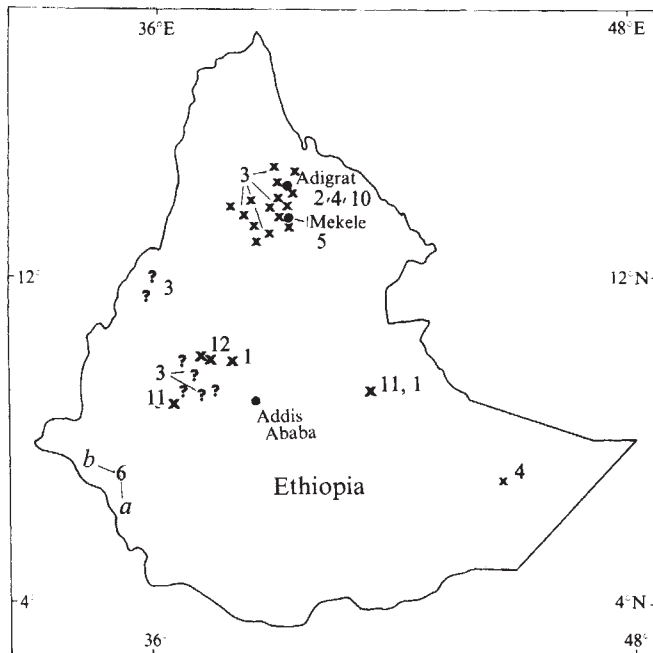


Fig. 1 Localities of post-Precambrian and pre-Jurassic (pre-Adigrat) strata in Ethiopia. a, Kari sandstone; b, Gilo sandstone; x, probable Karroo; x, pre-Adigrat; ?, Adigrat ?; numbers, references.

Several outcrop areas of apparently unfossiliferous fluvialite sandstones and conglomerates were discovered in western Kefa and Ilubabor Provinces, south-western Ethiopia, in 1974 during reconnaissance mapping⁶. The sediments are consolidated, tilted, and faulted; they lie unconformably on late Precambrian(?) crystalline basement and are overlain by mid to late Tertiary basalts with shallow angular unconformity. They differ distinctly in facies and thickness from the red, residual conglomeratic sandstone, generally less than 10 m thick and of unknown age, that marks the basement-basalt unconformity to the south-east^{6,13,14}. Samples suitable for palynological analysis, taken from two localities 125 km apart (Fig. 1, a and b), yielded identifiable Permian miospores. At locality a, the Kari sandstone, comprising at least 30 m of fluvialite sandstones with minor conglomerate and siltstone, occurs in several small outcrop areas, in part fault bounded, in which the strata dip gently south-westwards. Miospores are common, but most are corroded, broken, and dark brown to black, indicating a relatively high degree of thermal metamorphism of the organic material, and some post-depositional oxidation. On prolonged laboratory oxidation they became sufficiently translucent to allow identification of the following: *Alisporites* sp.; *Granulatisporites micronodosus* Balme and Hennelly; (?) *Horriditriteles ramosus* (B. and H.) Bharadwaj and Salujha; *Klausipollenites schaubergeri* (P. and K.) Klaus (Fig. 2c); *Kraeuselisporites* sp.; *Parasaccites* sp. (Fig. 2a); *Protohaploxylinus* sp. (Fig. 2b); *Striatopodocarpites* sp.; *Vittatina* sp. (Fig. 2d); and *V. (?) costabilis* Wilson. As the fossils were difficult to identify because of rather poor preservation, no information on the quantitative composition of the assemblage was obtained. On broad comparisons, the assemblage is like those from the Lower and Upper Permian of Gondwanaland in its relatively high percentage of *Striatiti*. It also resembles those from the Upper Permian of Europe in the presence of *Klausipollenites schaubergeri*. Thus, like Upper Permian assemblages of Gabon⁷, it seems to be somewhat transitional in composition between Gondwana and European assemblages.

At locality b (Fig. 1), the Gilo sandstone occurs in two

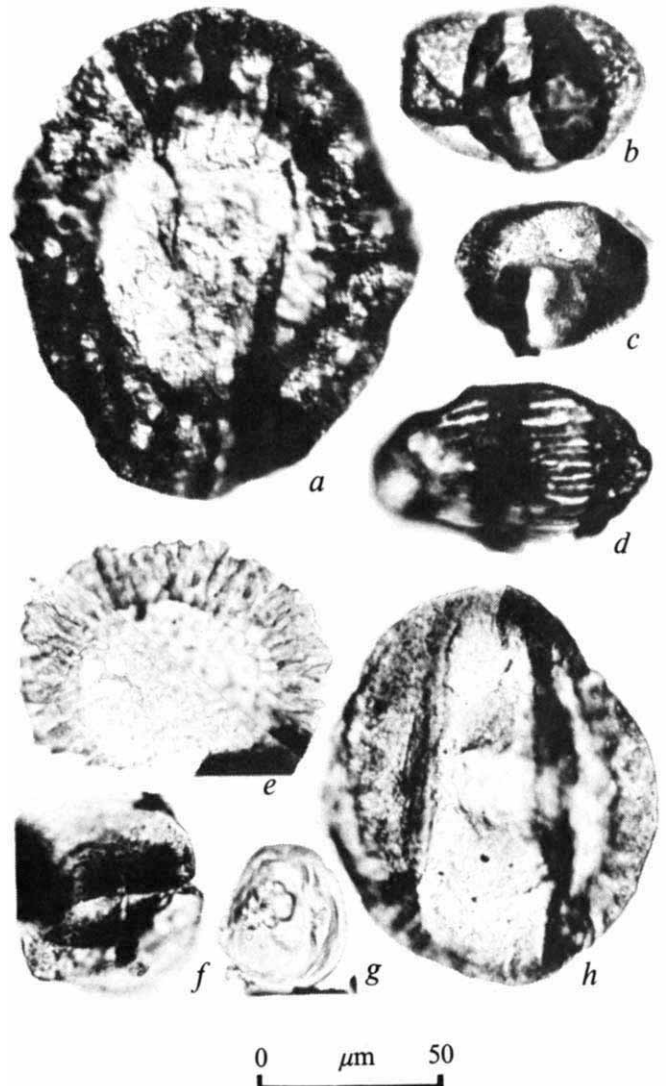


Fig. 2 a-d, Miospores from the Kari sandstone: a, *Parasaccites* sp. (GSC 45731); b, *Protohaploxylinus* sp. (GSC 45732); c, *Klausipollenites schaubergeri* (P. and K.) Klaus (GSC 45733); d, *Vittatina* sp. (GSC 45734). e-h, Miospores from the Gilo sandstone: e, (?) *Nuskoisporites gondwanensis* (B. and H.) (GSC 45735); f, (?) *Pallidosporites magnus* Schaar. (GSC 45736); g, (?) *Marsupipollenites* sp. (GSC 45737); h, (?) *Divarisaccus* sp. (GSC 45738). Numbers in brackets refer to specimens in the National Type Collection, Geological Survey of Canada, Ottawa.

basins separated by a north-westerly-trending basement arch. The poorly exposed south-western occurrence, 100 km² in area, is a homocline faulted against basement on its south-west side, and may contain as much as 1,500 m of strata, unless it is repeated by unrecognised faults. The north-eastern basin plunges gently north-westwards, and contains at least 300 m of strata, sparsely exposed within a 250 km² area. Calcite-cemented feldspathic quartz sandstones predominate, with interbedded arkosic conglomerate in the basal part. The transportation direction in both basins is mainly to the south-west. Miospores are relatively rare in the available sample from the north-eastern basin. In contrast to those from the Kari sandstone, they seem to have been little affected by thermal metamorphism, being brownish yellow without laboratory oxidation. The following were recognised: *Alisporites* sp.; *Cordaitina* sp.; (?) *Divarisaccus* sp. (Fig. 2h); *Kraeuselisporites* sp.; (?) *Marsupipollenites* sp. (Fig. 2g); (?) *Nuskoisporites gondwanensis* Balme and Hennelly (Fig. 2e); (?) *Pallidosporites magnus* Schaaarschmidt (Fig. 2f); *Parasaccites* sp.; and *Protohaploxylinus* sp. Most specimens are corroded, and this,

coupled with their relative rarity in the studied sample, makes precise age determination difficult. Therefore, as with the Kari sandstone sample, it is only possible to determine the age in broad terms from the palynological data. Striate pollen are rare, and large, radially symmetrical mono-saccate forms comprise a greater proportion of the assemblage than in the Kari sandstone, suggesting a closer affinity with Lower rather than Upper Permian assemblages of the Gondwana region^{8,15}. This suggestion is supported by the observation of a single specimen of (?)*Nuskoisporites gondwanensis* Balme and Hennelly (Fig. 2e; (?)=*Parasacites* sp. of Segroves⁹).

The limited miopore assemblages we have studied from the Kari and Gilo sandstones, although evidently Permian and similar to those of the Gondwana Province, can be cautiously dated more precisely as late and early Permian, respectively. Considering that the samples reported here were not collected specifically for palynological studies, however, it seems reasonable to predict that samples collected primarily for that purpose would give a better yield of palynomorphs and enable a more precise determination of the age and geological history of Permian strata in this part of Ethiopia.

In western central Ethiopia, 300–400 km north-east of the Kari and Gilo sandstones, large tracts of clastic sediments have been mapped as Adigrat Sandstone³, although pre-Adigrat sediments are known^{1,12} or suspected to be present locally. The term Adigrat Sandstone was at first applied collectively to sandstones lying in the same stratigraphic position in northern Ethiopia¹⁰, but later work^{2,5} has shown that parts of the original unit, namely the Enticho Sandstone, the Edaga Arbi Tillite, and the Enguya Sandstone, are older than the basal clastic sediments of the Mesozoic marine transgression, to which the name Adigrat Sandstone has now been restricted². The Adigrat Sandstone as redefined is Jurassic and is known to thin westwards beneath its cover of marine limestones⁴, although its rapid thinning to the west where overlying limestones are absent may be attributable in part to later erosion. On the basis of the occurrence of Permian strata in south-western Ethiopia reported here, and the recognition of pre-Adigrat sediments to the north-east, it seems likely that much of the apparently thick sedimentary succession mapped as Adigrat Sandstone in western central Ethiopia may also be of Karroo age. If so, Karroo sediments are more widespread north of their formerly recognised northern limit than was previously suspected.

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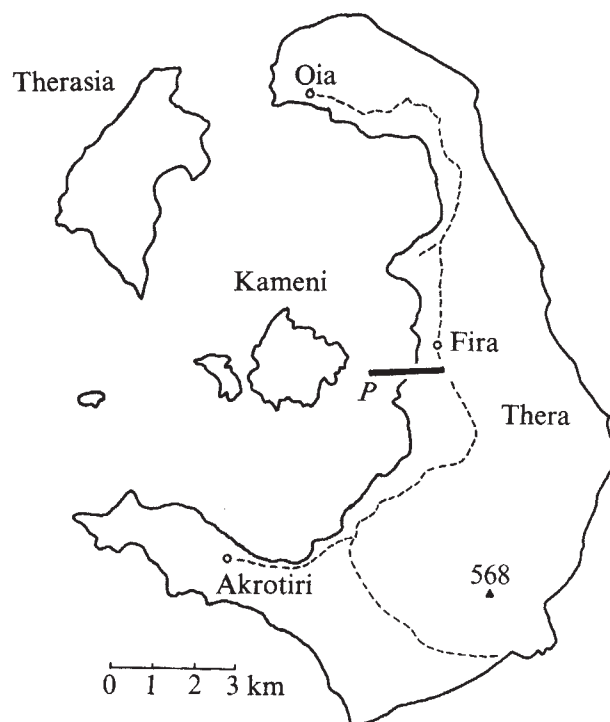
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Radiocarbon dates of Santorini volcanics

THE caldera walls of the ring-islands Thera and Therasia, belonging to the Santorini Group (Fig. 1) in the southern Aegean, are built up of lavas and pyroclastics, among which pumice layers are particularly striking (Fig. 2). The top layer (Bo, abbreviated from the German term “oberer Bimsstein”) comprises air-fall pumice and ashes, overlain by thick pyroclastic flows^{2,3}. It was produced by the catastrophic outburst of the Thera volcano in late-Minoan time, which culminated in the collapse of the Santorini caldera⁴. The decline of the Minoan civilisation around 1500 BC has been attributed to these volcanic events⁵. The Upper Pumice Series buried late-Minoan settlements on Thera and Therasia, and an important settlement near Akrotiri in the southern part of Thera is still being excavated⁶. The Upper Pumice Series rests on an old soil horizon (black layer in Fig. 2) in which remnants of late-Minoan houses were found in 1869 (ref. 7). Wood from such houses found at the base of the pumice quarries south of the town of Fira has provided radiocarbon dates of $3,370 \pm 100$ yr b.p. (ref. 8). This age coincides exactly with archaeological data for the destruction of late-Minoan settlements on Santorini⁹.

The Bo palaeosol is spread all over Thera and Therasia. Its thickness locally reaches 3–4 m, for example, on Therasia, where carbonised plants have been found in its lower parts. ¹⁴C dating of these plants has given an age of $12,950 \pm 756$ yr b.p. The palaeosol horizon rests unconformably on a small sequence of welded tuffs and slightly welded pumice flow deposits. A maximum thickness of 12 m for these ignimbrites occurs in the southern part of Thera. The walls of the partly excavated late-Minoan town near Akrotiri are founded on these ignimbrites. ¹⁴C ages of charred trees which sporadically occur in the pumice flow deposits forming the lower part of the ignimbrite series have provided ¹⁴C dates of $18,050 \pm 340$, $18,165 \pm 210$ and

Fig. 1 The Santorini Group in the Aegean Sea, Greece. The old ring-islands Thera and Therasia surround the late-Minoan Santorini caldera and the post-Minoan Kameni islands. P, location of the schematic profile (Fig. 2).



18,880±230 yr b.p. (ref. 3). The ages show that an exceptionally long interval of about 15,000 yr without any volcanic activity on Santorini preceded the late-Minoan outburst. During this interval the contemporaneous volcanic top layers were deeply decomposed and altered to a thick palaeosol.

The ignimbrite series is underlain by a stratigraphic sequence of at least 19 alternating pyroclastic strata comprising mostly dark ashes, cinders, scoriae, pumice, bombs, xenolithic lapilli, blocks, and so on. Many of these strata can be subdivided into numerous small beds and horizons. The total thickness of the complete sequence underlain by the Middle Pumice Series (Bm, "mittlerer Bimsstein") amounts to about 30 m. The sequence represents a long period of volcanic activity of the Thera and adjacent volcanoes with some overlap of their products. Intervals of quiescence are marked by some decomposed ash layers and fossil soil horizons, four of which are rich in plant fossils (*Chamerops*, *Olea*, *Pistacia*, *Phoenix*, and *Tamarix*). All of these genera still exist among Mediterranean flora. This means that during that time the Mediterranean climate was the same as at present.

Radiocarbon dating of a carbonised piece of tree found about 1.5 m under the lower plant horizon in the lower part of the pyroclastic sequence, has given an age of 36,700±1,025-950 yr b.p. (determination by the Niedersächsisches Landesamt für Bodenforschung, Hannover). Another ¹⁴C dating of the same sample (performed in the Geochron Laboratories, Cambridge, Massachusetts) has shown an age of more than 37,000 yr b.p. Therefore, the plant horizons of Santorini can be correlated with the Kalabaki I and II interstadial (between 37,000 and 35,000 yr b.p.) demonstrated by palynological studies near Thessaloniki in northern Greece⁹.

Twenty distinctive tephra layers, derived from various sources¹⁰, have been identified in Quaternary deep-sea cores of the eastern Mediterranean¹¹. Mellis¹² has correlated the upper tephra layer in the cores collected on the Albatross with the Upper Pumice Series on Santorini.

His correlation has been confirmed by Ninkovich and Heezen¹³, who have identified the Upper Tephra Layer in 14 cores collected on the Vema. The Middle Tephra Layer, which has been detected in Vema core 10-58, has been correlated with the Lower Pumice Series (Fig. 2, Bu, "unterer Bimsstein") on Santorini. Based on a ¹⁴C age of 17,860 yr b.p., taken from carbonised pumice found on the neighbouring island of Anaphi, the Lower Pumice Series was deposited¹⁴, probably 17,000-18,000 yr b.p. This is inconsistent, however, with the age of the Lower Pumice

Series if the plant horizons (37,000 yr) and their stratigraphic position (Fig. 2) are taken into account. According to that evidence, that part of the volcanic sequence on Santorini which underlies the plant horizons, including the Middle (Bm) and the Lower Pumice Series (Bu), must be older than 37,000 yr.

The Middle Tephra Layer of the Vema 10-58 core, however, can more appropriately be correlated with the ignimbrite series, for which an age of between 18,000 and 18,500 yr can be fixed. Mineralogical and petrochemical characteristics of the Middle Tephra Layer¹⁴ correspond exactly to the pumice in the lower part of the ignimbrite series. The refractive index of the glass is 1.514 (ref. 15), about the same as in the Lower Pumice Series. Therefore, the refractive index cannot be used as a discriminative factor¹⁴ between the two pumice series. It may also be noted that a layer in the Albatross core 189 has provided an age of 17,200±500 yr b.p. (ref. 16), which roughly fits with the age of the ignimbrite series.

Based on the fact that the Middle Tephra Layer is not to be correlated with the Lower Pumice Series but corresponds to the stratigraphically younger Ignimbrite Series, the position of the Lower Pumice Series in the deep sea cores is still unknown. In any case, the corresponding tephra layer must be situated below the "Ischia Tephra"¹⁴ (or "Lower Tephra"¹⁵), which has an age of 24,000 yr b.p.

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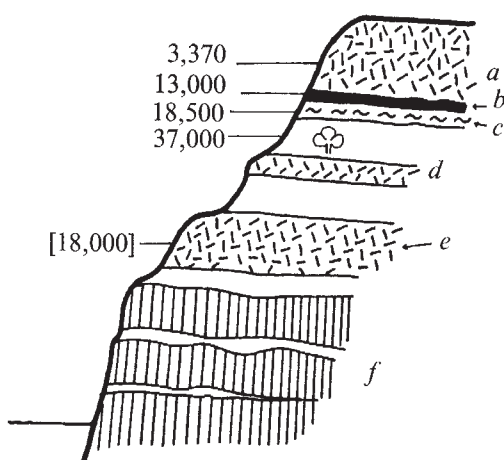
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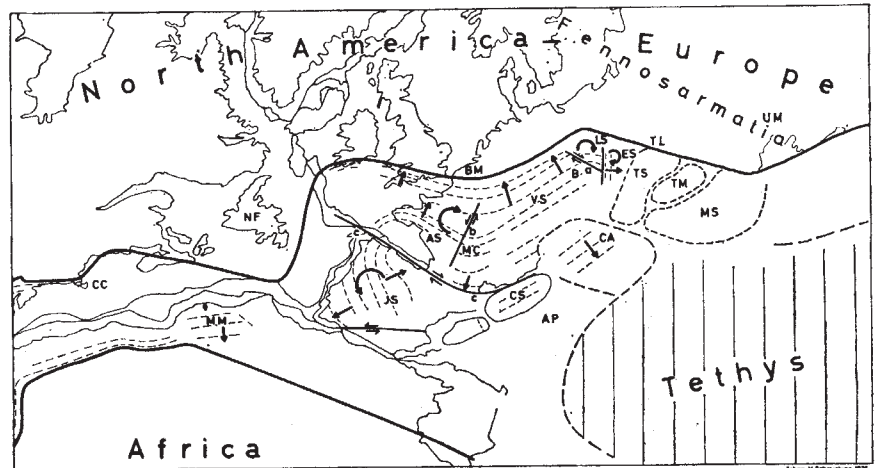
Fig. 2 Schematic profile (P in Fig. 1) of the caldera walls of Thera, south of Fira. For explanation see text. a, Upper Pumice (Bo); b, palaeosol; c, ignimbrites; d, Middle Pumice (Bm); e, Lower Pumice; f, lavas. Figures denote radiocarbon ages (yr b.p.).



Formation of Hercynian subplates, possible causes and consequences

WITH the exception of the microplate models of Badham and Halls¹ and Riding² models of Hercynian plate tectonics³⁻⁷ have assumed the existence of only one plate, that is, South Europe, between Africa and the Tethys in the south and North America-Europe and the Mid-European Ocean in the north. The South European plate has generally been considered as a stable unit. Studies of the Alpine

Fig. 1 Schematic map of the South Europe plate and neighbouring plates for the late Hercynian time showing subplates, major wrench faults, and general structural trends within South Europe. Base map after Bullard *et al.*²⁴ and Dewey *et al.*⁸. Thick lines = continental margins of bordering plates; thick arrows = direction of migrating fold-belts; thick curved arrows = direction of rotation of subplates in respect to Variscan subplate. Key to abbreviations: AP = Apulia; AS = American subplate; B = Bohemia; BM = Massif of Brabant; CA = Carnics; CC = Cape Cod; CS = Corsica-Sardinia; ES = East Sudetan subplate; IS = Iberian subplate; LS = Lugian-Silesian subplate; MC = Massif Central; MM = Moroccan Meseta; MS = Moesian subplate?; NF = Newfoundland; TL = Tornquist Line; TM = Tirgu-Mures subplate?; TS = Tatride subplate?; UM = Ukrainian Massif; VS = Variscan subplate; a = Elbe wrench fault; b = Sillon Houiller wrench fault; c = Biscaya-Pyrenees wrench fault.



system⁸, however, suggest that such an assumption may not be valid. I assume here that during the Carboniferous, South Europe was originally an elongated plate with nearly straight margins and internal structures. Collision with the irregular continental margins of the bounding plates North America-Europe and Africa induced subdivision of South Europe into semi-independent subunits, that is, into subplates, and formation of major arc-like structures and wrench faults.

Both North America-Europe and Africa were large plates. The postulated South Europe plate in contrast was characterised by great length and small but variable width compatible with an origin by accretion of new crust at an island arc system. In general, the oldest rocks of the plate occur within a central ridge that extends from Bohemia through the Massif Central, Brittany into Iberia and the Moroccan Meseta. Distribution of younger rocks to either side has been taken to indicate that two fold belts migrated away from the central ridge, one towards the north and the other towards the south^{7,9-12} (Fig. 1). This process has been ascribed to the oceanward movement of two opposed subduction zones^{9,13}. Metamorphism and emplacement of granites also began in the central ridge and became progressively younger towards the plate margins.

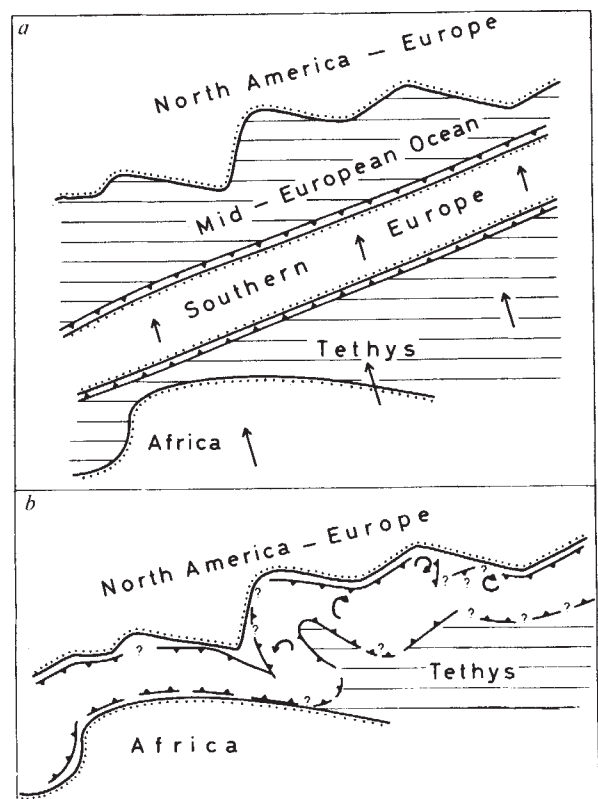
Dehydration and partial melting of former oceanic crust subducted beneath South Europe during the Carboniferous may have led to the formation of zones of hot partially molten upper mantle material above the opposed subduction zones. Diapiric uprise and lateral spreading of this material may have in turn caused widespread partial melting in the lower crust of South Europe and high temperature-low pressure metamorphism of upper crustal rocks¹³. As a consequence South Europe during the greater part of the Carboniferous was probably a rather thin and hot plate with a rigid crust ~ 20 km thick. In contrast the adjacent parts of both North America-Europe and Africa did not experience extensive granite emplacement and high temperature-low pressure metamorphism during the Carboniferous. It can therefore be assumed that the heatflow within both plates was lower than under South Europe and that both plates were cooler and consequently characterised by thicker lithosphere than South Europe. Applying this concept, which is of critical importance to the model given below, it is likely that the mechanical properties of the thin and hot South Europe plate were quite unusual and very different from those of the two major plates bordering it.

Uplift of the crust of South Europe, regional erosion of the central zone, formation of intermontane troughs and associated volcanism were also probably related directly to the uprise of hot upper mantle material. Spreading of this material caused migration in the onset of uplift, erosion, trough formation and volcanism¹³. This activity persisted

into the Lower Permian in spite of the cessation of subduction in Upper Carboniferous time because of the collisions between the South Europe plate and the bounding North America-Europe and Africa plates.

According to McKerrow and Ziegler¹⁴ and Dewey and Kidd¹⁵ the pre-Hercynian margin of eastern North America and southern Britain was formed by rifting during the Devonian when northern South America and North America-Europe collided and then separated again soon after. Subsequently, South Europe and Africa were sutured to North America-Europe by continent-continent collisions^{5,6,13}, events which also apparently caused the ter-

Fig. 2 a, Sketch map of the pre-collision South Europe plate with its two subduction zones (thrust lines), the bordering oceans and the irregular continental margins of North America-Europe and Africa. Arrows indicate potential direction of drift. b, Sketch map of the South Europe plate near the end of the continent-continent collisions. The South Europe plate adjusted to the irregular continental margins of the neighbouring plates by distortion, that is, formation of subplates. Curved arrows indicate sense of rotation of subplates.



mination of subduction of oceanic crust of the Tethys further to the east. The suture line, represented by Hercynian fold belts, is not straight, but probably reflects the pre-existing form of the North America–Europe continental margin. Assuming that the southern continental margin of North America–Europe underwent little modification during suturing it is likely that its previous form was irregular and of a configuration typical of rifted margins. There are projections and embayments. Long relatively straight segments meet at rather sharp bends in the manner described by Dewey and Burke¹⁶.

During its northward approach, South Europe eventually managed to fit into the irregularities of the southern continental margin of North America–Europe. South Europe is unlikely to have possessed the required configuration to fit the irregularities of the continental margin of the northern plate in the manner of a jig-saw puzzle. A more reasonable assumption is that the original configuration of South Europe was one of almost straight structural trends within the plate and also an originally straight northern plate margin (Fig. 2). This form would be favoured by an origin of the plate as a series of island arcs. During collision South Europe would have needed to adjust until it eventually fitted all irregularities of the southern continental margin of the North America–Europe assuming these were relatively unmodified by the collision. Progressive subduction of the Mid-European ocean crust between North America–Europe and South Europe would have allowed the latter plate to collide first with the southernmost projections of the former, then through internal deformation and wedging aside of large slices to advance further northwards and finally fit into the embayments of the continental margin of North America–Europe.

During this process, South Europe probably collided first with the Ukrainian Massif of Fennosarmatia. Along newly forming wrench faults subplates were driven aside and rotated, and were left behind by the main part of South Europe, which was able to proceed further northwards, west of the Tornquist line. Such subplates are the Lugian–Silesian and East Sudetan subplates, probably also the Hercynian massifs in the realm of the Carpathians, that is, the Tatríde, Tirgu–Mures, and Moesian subplates (Fig. 1). Palaeomagnetic studies of Upper Carboniferous rocks from the Bohemian Massif and the Inner Sudetic basin separated by the Elbe wrench fault indicate a late Hercynian rotation of 17° between these areas¹⁷. This relatively small rotation is believed to represent only part of the total rotation which is assumed to have commenced before the Westphalian. Evaluation of deep boreholes clearly shows that a postulated connection between the Rhenohercynian zone and Westphalian coal measures of the Ruhr district with equivalent zones of the Sudetic Mountains and the Upper Silesian coal basin¹⁸ does not exist¹⁹. This is believed to be caused by the disruption of the originally continuous fold belt during its formation by collision with Fennosarmatia. In spite of this disruption, later development of both areas followed similar paths, for example in the deposition of the Upper Carboniferous coal measures.

Another important bend in the southern continental margin of North Europe existed at the south-eastern margin of the Massif of Brabant, Belgium. South of this, nappe structures, unique in the Rhenohercynian zone, were probably developed in response to a local concentration of compressional forces. To the west of this bend there extended a large embayment bounded to the west by Newfoundland. It is postulated that the South Europe plate reacted to contact with this embayment by formation of several subplates. West of the Variscan subplate, the American and Iberian subplates formed, separated from each other by major wrench faults, that is, the Sillon Houiller and the Biscaya–Pyrenees wrench faults²⁰. The American subplate rotated clockwise relative to the Variscan subplate and

Iberia in turn rotated anticlockwise relative to both the Variscan and American subplates, these rotations, together with translations, finally resulted in the closure of the embayment. This process of rotation of both subplates in opposite directions would have caused intense shearing stresses and consequent intense internal deformation of both subplates. It is assumed that the partially molten and thus relatively soft lower crust could have reacted by plastic deformation recorded by the two large basement arcs in the Massif Central and in Brittany–NW Iberia whereas the more rigid upper crust could have reacted only through formation of many wrench faults within both subplates²⁰. Further analysis of the wrench fault system should clarify the mechanism by which the two subplates moved into the embayment.

Another consequence of this major tectonic event was that the south-eastern continental margins of both subplates which were originally colinear, were rotated until they finally came into contact. In addition, the two fold belts situated north-east of the Iberian ridge and south of the Massif Central were probably also initially colinear, and only during the later part of their evolution, that is, during collision of South Europe with North America–Europe, did they rotate to face each other, migrate towards each other and eventually come into contact in the general region of the Pyrenees. It seems quite possible that some crustal slices from the southern margin of South Europe were driven aside during this subplate collision. Van der Voo²¹ recently discussed palaeomagnetic data from some Palaeozoic Iberian rocks and concluded that Iberia rotated for at least 40°, that is the total value of the anticlockwise rotation of Iberia exceeds its Mesozoic value of 35°.

To the south-west of the large embayment, the Newfoundland projection and the bend in the continental margin near Cape Cod may have induced formation of major wrench faults, and thus the formation of several subplates, now located in Morocco, Algeria, and Southern Spain. In western Morocco, the general structural trend of the Hercynian fold belt is NE–SW to N–S. At the northern margin of the Moroccan Meseta, between Rabat and Tifet, however, there is a sharp bend in the structures towards the east^{22,23}. Varied structural trends in the Hercynian Massifs of NE Morocco and Algeria may in part be due to formation of Hercynian subplates. The Iberian and North African subplates must have been separated by major wrench faults across which large changes in the structural trends took place. Collision of the Moroccan, Algerian, and Iberian parts of South Europe with Africa probably also facilitated the squeezing of South Europe into the large Brabant–Newfoundland embayment.

During collision with elongated South Europe the irregular rifted continental margins of North America–Europe and Africa are believed, as argued above, to have induced formation of major arc-like basement structures and many wrench faults, some of which form the boundaries of subplates and thus to have produced the diversity in major structural trends of the Hercynian fold belt. The postulated mechanical properties of the South Europe plate, in contrast to those of the two large plates that flanked it, are believed to have enabled South Europe to react in the manner outlined. The force that drove South Europe against the flanking plates was apparently not as great as during collisions associated with the Alpine system. The scarcity of large nappes and lack of areas of strong post-collision uplift arising from one continental plate overriding another lend support to this model. A reason for this could have been a reduction in the velocities of the approach of the two large plates because of the spread of hot mantle material beneath the South Europe plate.

Late Hercynian formation of intermontane troughs, many of which are located on wrench faults, and associated volcanicity commenced in uppermost Viséan time. These late

Hercynian processes were very similar on the postulated subplates of South Europe, even in post-collision Lower Permian time. Thus crustal spreading of South Europe took place in a number of different directions simultaneously as the subplates in this respect behaved as individual units. This again seems to have been possible only if the force driving South Europe against the adjacent plates was not very great.

Two other possible consequences of the model are that the formation of the irregularly rifted continental margins of North America–Europe and north-western Africa might have influenced the pattern of subsequent opening in the relevant area of the North Atlantic, and ultimately may have played a part in the behaviour of South Europe during the Alpine cycle of plate tectonic processes. Some of the major wrench faults between the Hercynian subplates, being major zones of weakness may have been reactivated during the opening of the North Atlantic and during the Alpine cycle. Some of the subplates were probably blocked out and developed into microplates. Thus the heritage from the rifted continental margin of North America–Europe and the Hercynian plate tectonics may, in part, have caused some of the peculiarities of the Alpine orogenic belts, for example the numerous microplates described from the Mediterranean area⁸ and some of the arc-like fold belts as in the Carpathians.

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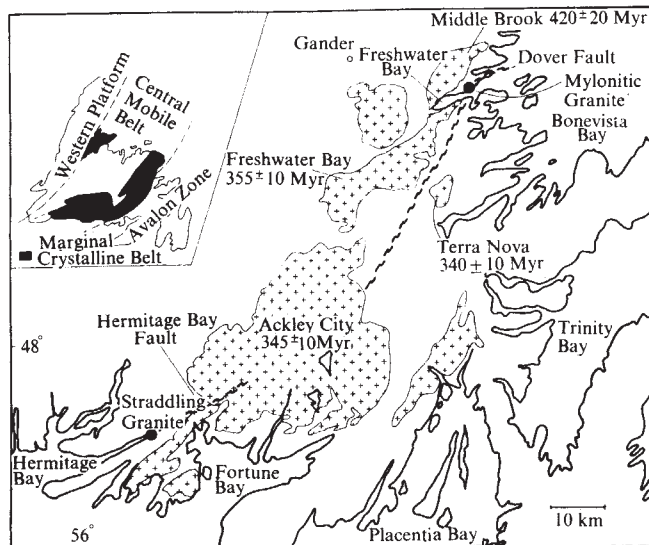


Fig. 1 Diagram showing the relationship of the Mylonitic and Straddling granites to the Hermitage Bay–Dover Fault System. The Rb–Sr age data are taken from ref. 8.

ocean in Cambrian and Lower Ordovician times, whereas the Western Platform (Fig. 1) was on the North American side of the ocean. The same evidence also suggested that the Proto-Atlantic ocean became narrower later in the Ordovician, although to what extent is very much a matter of debate. Wilson proposed that closure of the ocean and the consequent collision of the opposing continental margins was the mechanism responsible for much of the orogenic activity in Europe and eastern North America during Palaeozoic time, and that the opening of the present Atlantic resulted in transposition of fragments of the continents, such as is found in Newfoundland. Others (see ref. 2) have extended Wilson's original idea.

The site of closure of the Proto-Atlantic ocean may be marked in Newfoundland by the boundary between the Central Mobile Belt (Fig. 1) and the Avalon Zone^{3,4}, though alternative proposals have been made^{5,6}. The boundary separates a long strip of gneissic rocks and metasedimentary sequences (the Gander Zone) from the much less deformed, late Precambrian volcanics and sediments of the Avalon Zone⁴. The proposal has been made that the Hermitage Bay–Dover Fault System, a supposedly continuous fault system with a length of over 200 km, marks the boundary⁷. Unfortunately, a large part of it is obscured by the Ackley City Granite (Fig. 1), which has been dated at 345 ± 10 Myr (ref. 8).

Our geochronological evidence is provided by Rb–Sr, total-rock ages from two granites intimately associated with the Hermitage Bay–Dover Fault System. The "Straddling" Granite, at the head of Hermitage Bay, gives an isochron age of 490 ± 10 Myr (Fig. 2a), and the "Mylonitic" Granite, adjacent to the Dover Fault, gives an age of 400 ± 30 Myr (Fig. 2b). (Both intrusions have yet to receive formal names.) The analytical information is provided in Table 1.

The "Straddling" Granite, as its name implies, straddles the boundary between the Gander and Avalon Zones, and is cut by a breccia zone 50–100 m wide, which is the most southerly expression of the Hermitage Bay Fault. The pluton intrudes both the Garrison Hills Gneiss of the Gander Zone, and volcanics of the Connaigre Bay Group of the Avalon Zone. Although the granite is brecciated, (O'Driscoll, private communication) there is no sign of mylonitisation.

The "Mylonitic" Granite^{7,8} occurs adjacent to the trace of the Dover Fault, which is marked by a zone of mylonitisation 300–500 m wide and at least 20 km long⁷. The fault separates the Bonavista Bay Gneiss Complex (Gander Zone)

Age relationships along the Hermitage Bay–Dover Fault System, Newfoundland

We present here geochronological evidence that parts of the Central Mobile Belt and the Avalon Zone of Newfoundland have been contiguous since the Ordovician or earlier, and that some time after about 4 Myr, significant movement took place along part of the boundary.

Wilson¹ has used fossil, lithological, and palaeoclimatic evidence to suggest the existence of a Lower Palaeozoic Proto-Atlantic Ocean close to, but not coincident with, the site of the present Atlantic. The fossil evidence he cited showed that the Avalon Zone (Fig. 1) of the island of Newfoundland was on the European side of the Proto-Atlantic

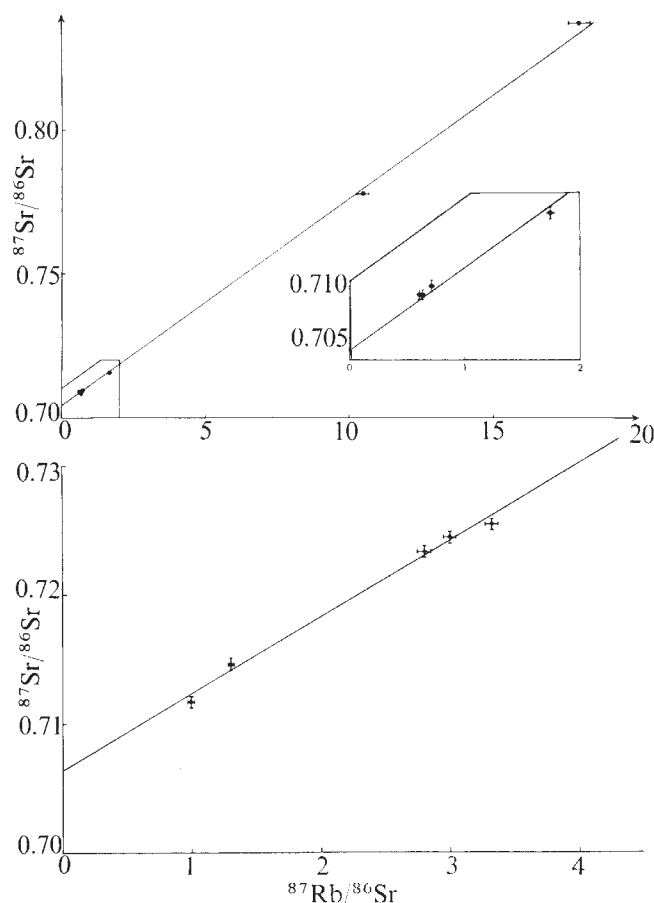
Table 1 Analytical results

Sample	Petrographic notes	Rb (p.p.m.)*	Sr (p.p.m.)*	$^{87}\text{Rb}/^{86}\text{Sr}$ (atomic)†	$^{87}\text{Sr}/^{86}\text{Sr}$ (atomic)‡
Straddling Granite	Medium to fine-grained granite, alkali feldspar mainly microcline, some perthite. Euhedral to subhedral plagioclase ($\sim\text{An}_{34}$), altered to sericite and epidote.	130	214	1.75	0.7158
SG-1		119	19	18.0	0.8367
SG-2	Anhedra quartz with some serrate boundaries, undulose extinction. Some polygonised grains. Accessory minerals: epidote, sericite, carbonate, sphene, apatite, chlorite	73	20	10.5	0.7779
SG-3		23	92	0.72	0.7094
SG-4		20	92	0.62	0.7087
SG-5		29	132	0.63	0.7087
SG-6					
Mylonitic Granite	Medium to fine-grained granite, alkali feldspar mainly microcline, some perthite. Granitic texture still evident in all but two of the samples. Plagioclase ($\text{An}_{21}\text{--An}_{32}$) altered and fractured, quartz usually anhedral, polygonised grains common, undulose extinction. Pronounced cataclastic deformation in MG-4 and MG-5; foliation defined by alternating bands of muscovite and fine-grained quartzofeldspathic material. Some feldspars augened, accessory minerals include sericite, epidote, chlorite and sphene	129	113	3.3	0.7255
MG-1		102	105	2.8	0.7234
MG-2		92	89	3.0	0.7245
MG-3		72	161	1.30	0.7146
MG-4		55	160	0.99	0.7117
MG-5					

* $\pm 2\%$
† $\pm 2\%$
‡ $\pm 0.1\%$ } at 99% confidence level; $\lambda^{87}\text{Rb} = 1.47 \times 10^{-11} \text{ yr}^{-1}$

from volcanics of the Love Cove Group (Avalon Zone); the latter are considered to be unconformably overlain by sediments of the Musgravetown Group (Avalon Zone). The granite exhibits a mylonitic fabric close to the fault, and there is some evidence for later brecciation. The age of 400 ± 30 Myr, we feel, represents the emplacement age of the granite and not the age of mylonitisation, since the samples dated were cataclastically deformed to markedly

Fig. 2 a, Rb-Sr isochron for the Straddling Granite: $t = 490 \pm 10$ Myr; $R_i = 0.7039 \pm 0.0006$; MSWD, 2.7. b, Rb-Sr isochron for the Mylonitic Granite: $t = 400 \pm 30$ Myr; $R_i = 0.7064 \pm 0.0012$; MSWD, 1.4. (Uncertainties given at 95% confidence level). (MSWD, Mean square of weighted deviates)



different degrees, yet still define an excellent isochron. Samples MG-4 and MG-5 (see Table 1) are the only true mylonites; the rest retain their granitic textures.

The isotopic data from the Straddling Granite are consistent with the geological evidence which suggests that the Garrison Hills Gneiss and the Connaigre Bay Group are both Precambrian^{10,11}. The significant finding, however, is that the southern portions of the Avalon and Gander Zones were in contact by the Ordovician, and it therefore seems extremely unlikely that the boundary between the Avalon Zone and the Central Mobile Belt represents the suture of the Proto-Atlantic Ocean.

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Upper limit to earthquake size

CHINNERY and North¹ have used data relating surface wave magnitude to seismic moment, M_0 , to convert magnitude-frequency statistics for the Earth into a seismic moment distribution. For M_0 values ranging from about 2×10^{25} to 2×10^{30} dyne cm they found that their data could be represented by

$$\log N = 17.47 - 0.61 \log M_0 \quad (1)$$

where N is the number of shallow earthquakes (depth < 70 km) each year with seismic moment greater than or equal to M_0 . On

Table 1 Data on relative plate motion (adapted from Kaula⁶)

Boundary type	Length (km)	Assumed width (km)	Mean transverse velocity (cm yr ⁻¹)	μ (dyne cm ⁻²)	Contribution to M_0 (tot) (dyne cm)
Ocean subducted under ocean	13,100	200	4.07	6×10^{11}	0.64×10^{29}
Ocean subducted under shelf	22,400	200	5.37	6×10^{11}	1.44×10^{29}
Ocean subducted under cordillera	10,700	200	8.32	6×10^{11}	1.07×10^{29}
Continent-continent collision	9,100	200	2.66	6×10^{11}	0.29×10^{29}
Oceanic fracture zone	16,900	5	1.32	3×10^{11}	3.4×10^{26}
Continent or island fracture zone	9,600	20	0.35	3×10^{11}	2.0×10^{26}
				M_0 (tot)	3.44×10^{29}

the basis of the remarkably good fit of equation (1) to their data, they argued that seismic moments considerably in excess of 2×10^{30} dyne cm were likely to occur as they did not consider it probable that the linear trend stopped immediately beyond the last data point. I have used recent results on the movement of lithospheric plates in conjunction with equation (1) to estimate an upper limit for M_0 . It turns out that this upper limit is probably not much in excess of the moment of the Chile earthquake of 1960 ($\sim 2 \times 10^{30}$ dyne cm) (ref. 2).

Nearly all of worldwide seismic deformation occurs at plate boundaries (see, for example, ref. 3). Most of the seismic deformation occurs in subduction zones and nearly all of the remainder in fracture zones. Brune⁴ and Davies and Brune⁵ have shown that seismic failure could account for essentially all of the relative motion at these plate boundaries. Accordingly, aseismic failure is assumed to be of little importance over the interplate boundaries defined by large shallow shocks.

According to Brune⁴, the total seismic moment each year, M_0 (tot), that is consistent with the relative motion of all of the tectonic plates is

$$M_0(\text{tot}) = \sum \mu_i D_i L_i W_i \quad (2)$$

where the summation is over all of the plate boundaries that are associated with large earthquakes. At each boundary, μ_i is the modulus of rigidity, D_i is the annual relative displacement, and L_i and W_i are the length and width, respectively, of the boundary. Because the material comprising the interplate boundaries has a finite strength, there can be no worldwide secular increase in elastic strain energy at these boundaries. Thus M_0 (tot) represents the average annual amount of seismic deformation that must occur to accommodate the observed interplate motion. Equating the total seismic moment to M_0 (tot) leads to

$$\int_0^{M_0(\text{max})} M_0 [-(dN/dM_0)] dM_0 = M_0(\text{tot}) \quad (3)$$

where the integrand is calculated from equation (1), and M_0 (max) is the maximum possible seismic moment. From equation (3)

$$M_0(\text{max}) = [M_0(\text{tot})/4.62 \times 10^{17}]^{2.564} \quad (4)$$

(Equation (4) is a statistical relationship in that equation (1) was based partly on worldwide magnitude-frequency statistics.)

To perform the integration of equation (3) it is necessary to assume that equation (1) is the correct distribution of M_0 up to M_0 (max). If M_0 (max) is not much in excess of 2×10^{30} dyne cm (the upper limit of the data presented by Chinnery and North¹) then this assumption is reasonable. From equation (4), then, an upper bound to earthquake moments can be calculated if M_0 (tot) can be estimated from the results of studies of the motions of plates of lithosphere. The calculation of M_0 (max), (equation (4)) does not depend on the time interval of one year, which was chosen for convenience.

Kaula⁶ presented data on relative plate motions in a convenient form for the calculation of M_0 (tot). Table 2 of his

paper lists the total lengths and mean transverse velocities of various types of interplate boundaries. His data for boundaries associated with large shocks are relisted here (Table 1) along with assumed widths for the zones of contact between plates, rigidities and the corresponding moments calculated from equation (2). The last column of Table 1 shows that in calculations of M_0 (tot) the contributions from the fracture zones are negligible.

The most critical assumption in the estimation of M_0 (tot) is the width of the zones of contact for shallow underthrusting. An average width of 200 km (Table 1) was chosen here on the basis of evidence reviewed by Kelleher *et al.*⁸. It does not seem likely that the average width of contact is less than 150 km or greater than 250 km.

From equation (4) M_0 (max) = 2.75×10^{30} dyne cm, which is not much greater than the moment of the Chile earthquake of 1960. For average widths of shallow underthrusting as little as 150 km or as great as 250 km the corresponding maximum moments (equation (4)) are 1.3×10^{30} and 4.9×10^{30} dyne cm. Thus, earthquakes substantially larger than the Chile shock of 1960 do not seem possible in view of the bounds associated with the relative motion of tectonic plates if equation (1), based on about 40 yr of seismic data, is the correct distribution of M_0 for the past 30,000 yr or more; the time resolution of the boundary rates in Table 1 is probably no better than 30,000 yr (Kaula, private communication). There is a possibility that the worldwide seismic-slip rate has decreased over the past 70 yr (ref. 5). This possibility introduces some uncertainty into the use of equation (1) for periods of time much greater than 40 yr.

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Ball lightning in Smethwick

BALL lightning has been observed on many occasions, but there have been few events¹⁻³ which have provided evidence with which to test any of the available explanations of the phenomenon. Consequently considerable weight has been placed on the "tub of water" event¹. The occurrence reported by Stenhoff⁶ has presented unique evidence which enables us to carry out a number of simple calculations. The lady concerned describes the fireball appearing 39-40" above the floor, then dropping 2 or 3" when she tried

to stop it with her hand. It was apparent for ~ 1 s and had a diameter of $\sim 4''$ but also had a halo.

The position of the hole in the dress is consistent with the fireball being ~ 700 mm above the floor when it struck. The hole is 100×70 mm, but the narrowest part indicates that the core of the fireball was ~ 20 mm in diameter. The surface of the fabric had melted over an area about 300 mm across.

The material seems to be a polyester which would melt at 250°C and have a specific heat capacity of $1.2 \text{ J g}^{-1} \text{ K}^{-1}$. To melt the material where there is now a hole would require 200 J and to melt the surface to a depth of 0.1 mm, 240 J. Even if this were only 10% of the total energy, it is $< 10^{-2}$ of the values quoted previously. Very little charring had occurred.

An estimate may be made of the energy radiated by the fireball since the lady said that "she seemed to glow all over" as the fireball approached. Assuming this sensation to be equivalent to a 1-kW radiator at a distance of 0.5 m, the flux would be 0.6 kW m^{-2} . A body area of 0.6 m^2 would receive 400 J in 1 s. An equivalent power would be radiated by a spherical black body of 10 cm diameter at 950°C or at $3,500^\circ\text{C}$ for a diameter of 1 cm. The thermal energy contained in the sphere would then be 2 kJ. In a dense plasma, radiation trapping reduces the rate of loss of energy and thus implies a higher temperature¹⁰. Since the ball did not convect rapidly, its mean density must have been close to that of the ambient air, indicating a confining force which if magnetic would require a field $\sim 1.5 \text{ T}$. It is interesting to note that Brand⁷ concluded that there was no heat effect with "the type of ball lightning that floats free in the air"⁸.

It has been proposed that ^{15}O and ^{17}F are formed during a lightning discharge^{9,10} but their half lives are 2.1 min and 66 s respectively, so there was no point in measuring β activity when the dress became available for examination 10 weeks after the event. A γ spectrum taken over a period of 1,000 min for energies from 0 to 3.6 MeV produced a total count over 400 channels of 1.1 c.p.m. above background, implying an activity of 1.6 pCi. No signs of chemical reaction were apparent.

The lady is alive and healthy, so we know that she has not been subjected to harmful ionising radiation. Taking a figure of 500 rad as the maximum exposure that may be suffered without apparent physiological effect, an upper limit of 250 J may be placed on the whole body radiation. This energy would be produced by the annihilation of 10^{-11} g of antimatter at a distance of 10 cm, a figure which is $< 10^{-2}$ that discussed in an antimatter theory of ball lightning⁵.

The burn suffered in the vicinity of the gold wedding ring permits another estimate of energy to be made. The burn was described as being lighter than a scald, so it is assumed that the skin temperature rose no more than 100°C . About 3 kJ would heat the surface of the hand ($10 \times 10 \text{ cm}^2$) to a depth of 1 mm. To heat the ring up to 100°C by an electromagnetic field¹¹ would require a current of 10^3 A and imply an electric component of the e.m. field of 10^4 V cm^{-1} . The resonant frequency would be 10^9 Hz , assuming an inductance of 10^{-9} H and capacitance of 10^{-11} F . Although large d.c. electric fields are present during thunderstorms, only small electromagnetic fields at GHz frequency have been measured¹².

These orders of magnitude 'guesstimates' suggest that the energy associated with this fireball is $\sim 10^{-3}$ of that calculated previously¹³. Nothing can be found to support the theories based on radioactivity, antimatter or magnetic fields. Furthermore, the low energies estimated are inconsistent with the assumptions on which some models of ball lightning are based.

I am grateful to Miss Stephanie Pearson for drawing my attention to this event, to the lady who suffered the alarm-

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Non-crystallinity of ion-irradiated metals

THE preparation of various metals and metallic alloys in a non-crystalline state using splat cooling, vapour quenching or electrochemical deposition techniques has been reported by many authors. A distinguishing characteristic of such amorphous films is a severe modification of the structure factor from that of the crystalline state. Although usually formed only at low temperatures the non-crystalline state may in some cases be metastable to above room temperature, this being particularly true for alloys near the eutectic. We examine here evidence for metastable non-crystalline alloys resulting from ion irradiation.

The structural modification of metals by ion irradiation has traditionally been treated in terms of replacement collision sequences or thermal spikes followed by a return to crystallinity with the freezing-in of point defects. At high ion doses the agglomeration of point defects may occur, but the final structure is considered to be that of a defected crystal. This is in contrast to the behaviour of ion-irradiated semiconductors, in which demorphisation occurs. Since, however, each collision cascade causes the temporary displacement of a large number of metal atoms and energy dissipation is rapid, the situation locally may well be compared with splat cooling, and the formation of a non-crystalline state in suitable cases might be anticipated. Stephens *et al.*¹ have reported that Rutherford backscattering and channelling analysis of Dy^+ implanted into single-crystal Ni produced a disorder peak structure which is uncharacteristic of metals, but which can usually be obtained with semiconductors rendered amorphous by ion bombardment. The Dy-implanted Ni system was consequently chosen for more detailed transmission electron microscopy examination, the results of which are reported here.

In our experiments, high purity Ni samples prepared for transmission electron microscopic examination were irradiated at room temperature with 20 keV Dy^+ ions to doses of $10^{16} \text{ ions cm}^{-2}$, thus giving a peak implant concentration of $\sim 30 \text{ atom \%}$ as confirmed by Rutherford backscattering. Analysis using electron diffraction in the TEM shows, in addition to Ni matrix reflections attributable to the undamaged crystal lying beyond the ion range, a single diffuse scattering maximum with a peak intensity scattering parameter $s = 2.99 \pm 0.01 \text{ \AA}^{-1}$, and a half intensity width $\Delta s = 1.1 \pm 0.2 \text{ \AA}^{-1}$. This should be compared with the value $s = 3.085 \text{ \AA}^{-1}$ for $\langle 111 \rangle$ Ni. Dark field images using the diffuse ring show a generally high background intensity with occasional resolved crystallites in the 30-100 $\text{\AA}$ range. The former should be expected since application of the Scherrer particle size broadening equation to the measurements quoted already indicated particle dimensions of $< 10 \text{ \AA}$, which is below the resolution limit of the

microscopy. On annealing above $\sim 400^\circ\text{C}$ the diffuse ring sharpens and widens to a good Ni $\langle 111 \rangle$ fit concurrent with the appearance of additional sharp rings attributable to higher order Ni reflections. Dark-field microscopy confirms the presence of large numbers of Ni crystallites 50–200 Å in size.

Various models of amorphous solids based on the random packing of hard spheres with no appreciable order beyond first neighbours have been proposed; an alternative model for a quasi-amorphous solid is a microcrystalline arrangement in which isolated groups of atoms adopt the parent crystal structure but are separated by localised energy barriers to recrystallisation. In particular, Wang and Merz² have proposed a microcrystalline arrangement in which atomic groups may take up the crystal structure of any one of a number of polymorphic forms, and have correlated the occurrence of non-crystallinity with bonding anisotropy and polymorphicity. According to this classification, Ni, with three polymorphs, should be one of the least structurally stable metals. For very small crystals the difference between a microcrystalline solid and a true amorphous solid becomes increasingly indistinct, although it is interesting to note that the observed increase in nearest-neighbour distance in our experiments has also been observed by Davies and Grundy³ in amorphous Ni films where a dense random packing model seemed justified.

Numerous authors have noted the enhanced stability of non-crystalline metals when alloyed with other metals or metalloids, and Nagel and Tauc⁴, using a nearly free electron approach, have suggested that this stability is attributable to a shift in the effective valency, causing the Fermi level to lie at a dip in the density of states function. Thus, an alloy with concentration x of element valency z_2 in z_1 should be most stable against recrystallisation when

$$1.7 \approx z_1(1-x) + z_2x$$

Substitution of $z_1=1$ for Ni, which as a group VIII element may be treated as having only one free electron per atom in the liquid, and $z_2=3$ for Dy gives $x=35\%$, similar to the peak concentrations achieved in the implantations reported here.

Of interest here are the data of Cullis, Poate and Borders⁵ who have observed a diffuse diffraction maximum following ion implantation of W^+ into Cu at concentrations in excess of 10 atom%. This figure may be compared with a most stable concentration of 14 atom% predicted by the equation given here.

Finally, we have noted the complete absence of a non-crystalline phase in Pb^+ irradiated Ni up to concentrations of ~ 35 atom%, where $z_2=2$ gives $x=70\%$.

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Effect of roughness on rubber friction when waves of detachment are present

IN some circumstances, when rubber moves over a hard surface no true sliding occurs. Instead, the contact area is crossed by waves of detachment¹; and it is only in these regions, where contact has temporarily been lost, that there is any relative motion of the two surfaces. The effect resembles the motion of a caterpillar on a leaf. It is now well established that when an elastometer slides over a smooth surface in these conditions, the frictional force may be accounted for in terms of the net work required to peel the rubber away from the counterface and readhere it^{2,3}. We have now found that waves of detachment may be present when the counterface is rough, and that the same explanation of the friction force applies.

The adhesion of rubber depends markedly on the roughness of the surface with which it is in contact^{4,5}. Slight roughness can increase the adhesion compared with an ideally smooth surface, while larger values of roughness can greatly reduce it⁶. When a cylinder rolls on clean rubber, the two surfaces are adhering together at the front of the contact and peeling apart at the rear. The net work for this process at a given velocity may be measured by rolling cylinders down an inclined plane.

For the friction experiments, silicone rubber was moulded to be optically smooth. The counterface was Perspex, which was roughened by blasting with fine particles. The two surfaces were brought together in a crossed cylinders configuration with a sliding speed of 1 mm s^{-1} . The frictional force was recorded, and the waves were filmed at $200 \text{ frames s}^{-1}$.

If Γ is the net work required to peel apart and readhere unit area, A the observed area of contact, and f the number of waves crossing the interface per s; then the energy required in 1 s may be equated to the work done by the frictional force F moving at the sliding speed V thus

$$FV = \Gamma f A$$

Thus, a value of Γ may be deduced and compared with Γ measured directly from rolling experiments for the same surface at the velocity of the waves. This is done in Table 1, and the agreement is fairly satisfactory for a friction experiment.

Table 1 Correlation between the adhesion energy required to account for the frictional force in terms of waves of detachment (Γ_1), and the adhesion energy deduced directly from rolling experiments (Γ_2)

Ra (μm)		F (N)	f (s^{-1})	V (mm s^{-1})	Γ_1 (J m^{-1})	Γ_2 (J m^{-2})
0.04	*	0.156	5.09	10.92	2.40	2.21
0.05	*	0.157	5.49	10.56	2.24	2.14
0.16	†	0.157	4.79	12.42	2.57	3.72
0.30	†	0.164	4.88	10.38	2.63	4.16
0.95	†	0.164	3.30	8.92	4.01	4.81
1.00	†	0.165	4.08	9.31	3.17	5.03
1.29	†	0.162	3.58	9.90	3.54	5.03
1.73	†	0.167	3.50	9.60	3.73	3.72
2.38	†	0.161	3.32	10.22	3.80	4.16
3.09	†	0.168	4.72	8.56	2.79	2.84
3.14	†	0.162	4.24	9.74	2.99	3.94
6.46	‡	0.179	4.96	10.94	2.82	2.63
7.87	‡	0.186	6.15	10.71	2.37	1.74
8.79	§	0.187	7.05	10.54	2.08	1.93
9.20	‡	0.184	6.05	10.17	2.38	1.36

The normal load was 0.1 N and the imposed sliding speed 1 mm s^{-1} . The results are for a range of roughness measured in terms of the centre line average (Ra). F is the frictional force, f the number of waves crossing the interface per second, and V the mean wave velocity. The rubber was Dow Corning XF-13-523 dielectric gel. The surfaces were prepared by blasting with fine particles as follows: *, untreated; †, glass beads; ‡, metal particles; §, sand. The correlation coefficient for the last two columns is 0.79.

At the highest roughness it became difficult to see exactly where contact existed. Waves were clearly observed to propagate, but it may be that with the low true contact area here there is also some interfacial sliding.

It is interesting to note that the coefficient of friction does not vary markedly with roughness. This is because of two competing factors. A Burgers vector, b , may be defined for a wave as the interfacial displacement which results when it passes. The total displacement per s is

$$V = fb$$

When Γ increases, bigger waves form, and they have a larger Burgers vector. Therefore they do not form so often and f decreases, so that F does not change very much.

It has similarly been observed that F may be insensitive to change in V , even though Γ is markedly velocity dependent. This may represent a general result for rubber friction when waves of detachment are present, that although the frictional force is to be accounted for in terms of the net adhesion energy Γ , changes in Γ may have little effect on the coefficient of friction for a particular elastomer.

A theoretical account, which is in preparation, provides a more detailed explanation of these observations. We are grateful to Professor D. Tabor for help and encouragement, and also to the SRC for an Assist award to G.A.D.B.

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Learning-like phenomena in stereopsis

WHEN confronted with Julesz random-dot stereograms¹ for the first time, naive observers often need as long as a minute or more to see a global figure in depth. With repeated exposure to the stereograms this perception time is progressively reduced until they begin to see depth almost instantaneously². This acceleration of the stereoscopic process over successive trials is a remarkable example of perceptual learning^{2,3}, and unlike many other kinds of perceptual learning it seems to be a purely visual effect. Further, stereopsis is a basic visual function for which much of the processing may occur as early as the prestriate cortex⁴. It is important to note that learning-like changes can affect such a process, and knowledge of elementary visual function may be helpful in determining just what is learned. Here I explore the position specificity of stereo-learning, and find that in most observers a significant proportion of the learning that is acquired in one retinal area does not transfer to neighbouring areas. One question raised by this finding is whether such position specificity necessarily implies localisation of the learning process to specific anatomical areas in the brain.

The stimulus used (Fig. 1) was a Xerox of Fig. 4.5–3 from Julesz's book¹. The global figure portrayed (Fig. 3) was a 'saddle' with a 'doughnut' threaded through it (hyperbolic paraboloid with torus) and the pattern was composed of $1,000 \times 1,000$ disparity elements, of which 90% were white and 10% black. The maximum disparity was in the range of 30'.

The stereogram was displayed through a three-channel

tachistoscope (Electronics Developments). The two half images were carefully aligned before the experiment so that the surround had zero disparity, and crossed polaroids were used to separate the two eye pictures. The whole stereogram subtended 9° at the viewing distance used.

A tiny yellow fixation rectangle was displayed (to both eyes) through the third channel of the tachistoscope. This ensured that the observer was converging on the plane of zero disparity in the stereogram. The stereogram was mounted in such a way that the fixation spot occupied a position between the 'saddle' and the 'doughnut' portions of the global figure as shown in Fig. 2. This will be explained below in greater detail. During the experiment the observer was asked to fixate this spot and never to move his eyes away from the spot. He was also encouraged to move his eyes very slightly within the spot to prevent the image from fading.

Each trial began with the exposure of the fixation rectangle. The subject initiated the exposure of the patterns by pressing a button on command which also started a timer. He was instructed to hold the button pressed until he could clearly see the curved three-dimensional surface of the saddle surrounded by the doughnut. A pencil sketch of the figure was shown to him. It was emphasised that he should use a constant criterion for reporting the emergence of the figure. His release of the button stopped the timer and recorded his perception time on that trial. The button release also switched off the stereogram. There was then a 10-s interval before he was required to initiate the next trial.

Trials were begun with the 'saddle' portion of the global figure occupying a position either to the right (seven observers) or to the left (seven observers) of the fixation point. They were continued until the perception time reached an asymptotic value. After the normal intertrial interval the whole stereogram was shifted by 2.5°, so that the saddle now came to lie on the opposite side of the fixation spot. To ensure that the observer knew exactly where to expect the global figure he was told that the stereogram had been shifted. The time course of his learning was then followed over a number of trials for this new position to the stereogram.

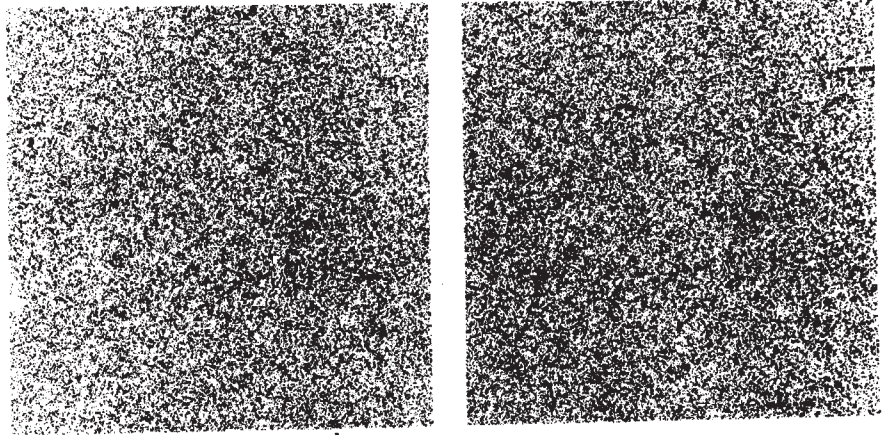
Fourteen subjects were used and all were naive as to the purpose of the experiment. Some of them had seen the 'standard' Julesz stereogram before, but not complex three-dimensional surfaces.

In Fig. 3 the perception time is plotted as a function of trial number for each subject. The line joining the points is broken where the stereogram was moved to a new position. The graphs clearly show the decrease of perception time over trials with the initial position of the stereogram.

Change in position produced an increase in perception time for 13 of the 14 subjects. In most of these 13 cases the time on the first trial for the new position was far outside the range of the preceding asymptotic trials. On subsequent trials there was a steady decline in perception time usually down to the original asymptotic value. In at least nine of the observers the magnitude of change was large (20–50 s change from the original asymptotic level) and a 'criterion' shift seems very unlikely. Most of them, on being questioned, reported unhesitatingly that they had not in fact seen anything during the long latent period on the first trial for the new position of the stereogram and that they had seen the figure emerge only just before button release. These results suggest that a significant proportion of the learning that is acquired in a particular retinal area, remains confined to that area.

When interpreting this lack of transfer, however, the possible role of nonspecific 'distraction' effects must be considered. The involvement of such effects seems unlikely for the following reasons. The same result was obtained on four additional subjects when a 'distributed practice'

Fig. 1 Random-dot stereogram composed of $1,000 \times 1,000$ disparity elements portraying a cyclopean hyperbolic paraboloid with torus. If the reader has not seen random-dot stereograms before, he will notice the long latency before the stereoscopic figure emerges (about 1.5 min; average for 20 subjects). With six or seven exposures to the same pattern the perception time is progressively reduced to just 1–2 s. Once the figure has been seen, wide conjugate eye movements may be made without 'losing' the global percept. It would seem that although the learning *per se* is position specific, the solution to the global stereopsis 'problem' is invariant with respect to spatial location.



paradigm was used extending over several days. 'Long term' stereo learning was found to be position specific to the same degree as the short term effects reported above. The learning is not specific to the particular random sequence of dots used. Changing the random sequence without shifting the global figure resulted in complete transfer of learning.

The role of eye movements in stereo learning also remains to be elucidated. Testing an additional eight naive subjects, I found that as soon as they had become skilled, seven of them could see complex three-dimensional surfaces (for example, Fig. 1) in 150-ms flashes. When the stereogram was then shifted to a new position, these eight subjects showed the same basic relearning effect as the 13 subjects in Fig. 3. It seems likely, therefore, that at least some component of stereo learning occurs in the visual centres independent of eye movements. But this point needs to be confirmed using stabilised images.

Fig. 2 Sketch of the cyclopean figure which was shown to the observers, illustrates the way in which the figure is shifted in relation to the fixation spot.

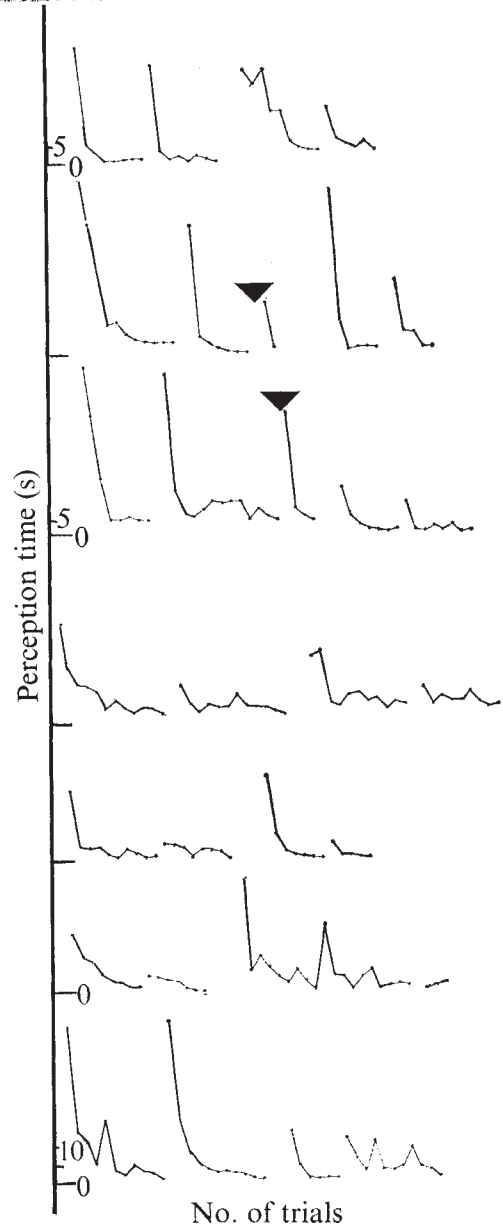
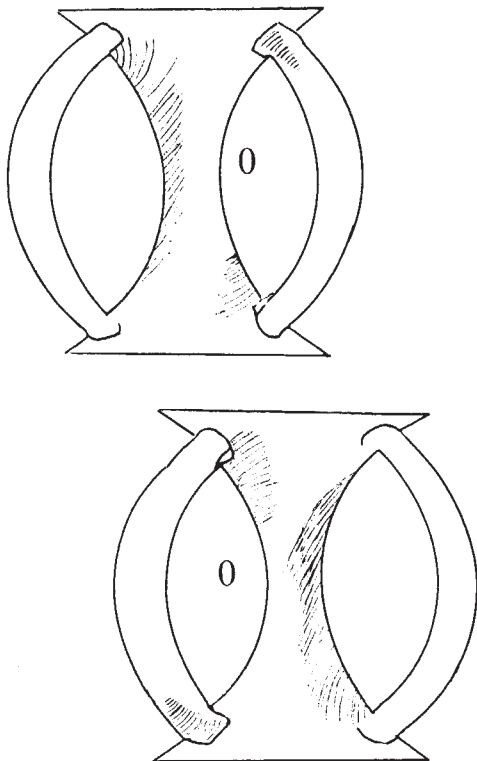


Fig. 3 Perception time on successive trials for 14 subjects. Each point refers to a single trial. On some trials the perception time was too long to be included in the graph; for these the line ending at the top of the plotting area points to the position of the data point. Data for each subject plotted to the same scale. The line connecting the dots is broken where the pattern was shifted between trials. Two of the subjects (*c* and *e*) were switched back again to the original position of the stereogram after they had reached an asymptote on the second position: ▼, point where this change was made. They apparently retained very little of the training they had acquired on the first position.

It is possible that what the observer initially learns is a strategy of vergence movements to bring disparate portions sequentially into register. As the observer becomes more skilled, overt eye movements are eliminated and the 'perception' of global stereopsis may simply involve a subliminal activation of the 'phase sequence' of sensory and motor elements that were originally driving the vergence system. This is equivalent to saying that vergence is replaced by 'cortical shifts' or that the Panum areas are permanently 'extended' by training. This line of reasoning is similar to that used by Hebb⁶ to explain form perception.

Visual memory is generally invariant with respect to location in the visual field^{7,8}; some authors have pointed out analogies between holograms and memory and suggested that the brain may store spatial Fourier transforms of visual inputs⁹. From my finding, however, that stereo learning is position specific, as well as from the observations of Wallach and Austin¹⁰, it may not be too fanciful to suggest that at least some forms of visual memory are encoded and stored in specific brain areas in such a way as to become unavailable to neighbouring areas.

There is obviously considerable scope for further experiments in this field. Most conventional learning paradigms—such as massed compared with distributed practice, 'forgetting', relearning, and so on—could be fruitfully studied using Julesz patterns as stimuli. Such studies could provide novel insights into mechanisms underlying perceptual memory and learning.

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Counter-intuitive property of effective population size

CONSIDER an organism with both autosomal and X-linked loci. The effective population sizes for an autosomal (N_{ea}) and an X-linked (N_{es}) locus can be shown¹ to be

$$N_{ea} = 4MF/(F+M) \quad (1)$$

$$N_{es} = 4.5MF/(F+2M) \quad (2)$$

where M and F are, respectively, the numbers of reproducing males and females in a random-mating population.

The ratio of the X-linked effective population size to that for autosomal loci is, from equations (1) and (2)

$$N_{es}/N_{ea} = 9(F+M)/8(F+2M) \quad (3)$$

From (3), X-linked loci have only 75% of the effective population size that autosomal loci have when males and females are equally abundant, and the discrepancy increases when males outnumber females, as seems intuitively obvious. When there are seven females for every male, however, the two types of loci have the same effective population size; when the proportion of females is increased further, X-linked loci have the greater effective population size. The asymptotic values approached by the ratio N_{es}/N_{ea} are 9/8, as the number of

females per male increases, and 9/16, as the number of males per female becomes infinitely large.

The biological significance of the above observation is hard to evaluate, but two points can be made. First, many insects, particularly parasitoids, have extremely female-biased sex ratios, and these species are mostly male haploids². Second, it is now possible to test the influence of effective population size on genic diversity, in that preliminary evidence³ indicates that male haploid and X-linked loci have fewer polymorphisms than autosomal loci. If this difference is general and effective population size differences are the major cause for it, then it should be still greater in male-biased male diploids, but in highly female-biased such species' X-linked loci should be more polymorphic than autosomal ones.

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Geography of photoperiodic response in diapausing mosquito

IN temperate latitudes, many arthropods rely on photoperiod to cue their seasonal development. Although the relationship between critical photoperiod and latitude is well illustrated, that between critical photoperiod and altitude remains obscure^{1,2}. I wish to consider photoperiodic control of dormancy in the pitcher-plant mosquito, *Wyeomyia smithii*, and quantify the relative effects of altitude and latitude on the photoperiodic response of an organism.

W. smithii is found from the Gulf coast to Labrador and northern Manitoba^{3–6} where it confines its breeding site to the water-filled leaves of a single species of plant, *Sarracenia purpurea*. The larvae overwinter in the pitchers in a state of developmental arrest. Short days evoke and maintain, while long days avert or terminate this diapause^{7,8}. The photoperiod which promotes 50% development and initiates or maintains 50% diapause is known as the critical photoperiod^{9,10}. In *W. smithii*, the critical photoperiod is the same for the initiation and termination of diapause among larvae reared in the laboratory and for the termination of diapause among larvae collected during early autumn⁷. I have collected larvae north of 38°N latitude only during the autumn (autumn larvae) and south of this latitude only during the winter (winter larvae). I packed the autumn larvae in ice on the day of capture and maintained them on ice until they reached the laboratory where I stored them in an ordinary refrigerator at 4±2 °C until the start of experiments. All experiments were begun within 45 d of capture. Since the winter larvae received unknown chilling in nature which might have affected the critical photoperiod^{11,12}, I reared them in the laboratory and determined the critical photoperiod for the initiation of diapause in the F₁ generation. Whether considering the maintenance of diapause among autumn larvae or the initiation of diapause among the F₁ of winter larvae, I exposed them to a range of LD 10 : 14 to LD 17 : 7 in 30-min increments at 25±1 °C. Rearing procedures and the criteria for diapause and development were the same as in previous studies^{7,13}.

I determined the critical photoperiod for 22 populations

of *W. smithii* collected over a range of 19° latitude, 20° longitude and 1,200 m altitude. The critical photoperiods ranged from 12 to 16 h of light per day (Fig. 1) and were closely correlated with latitude ($r=14.26$; $P<0.001$) and altitude ($r=9.25$; $P<0.001$) but not longitude ($t=1.71$; $P>0.10$). Latitude accounted for 80.5% of the variation in critical photoperiod and altitude an additional 15.5%. Critical photoperiod may then be estimated from latitude and altitude by the least-squares regression equation

critical photoperiod = $6.51 + 0.185 \text{ latitude} + 0.00129 \text{ altitude}$

To correlate critical photoperiod with climatic variables, I modelled the growing season (mean number of freeze-free days) for 26 cities in the United States north of latitude 30°N and east of longitude 90°W (ref. 14). Although city temperatures are not the same as in bogs, they are presumably correlated¹⁵. I found that growing season was closely correlated ($r=0.97$) with latitude ($t=15.39$; $P<0.001$) and altitude ($t=4.59$; $P<0.001$) but not longitude ($t=0.42$; $P>0.60$). I then calculated the growing season for each locality in Fig. 1 using its latitude and altitude and the least-squares regression equation relating growing season to these parameters

growing season = $585.93 - 9.33 \text{ latitude} - 0.094 \text{ altitude}$

I found that growing season calculated in this manner was an excellent predictor of critical photoperiod in *W. smithii* (Fig. 2). This degree of correlation between an ecogeographic character and a specific climatic variable is unprecedented. In many other studies¹⁶⁻²⁰, the ecogeographic characters being examined are affected by a wide range of environmental influences which may be changing independently of geography. The photoperiodic response of *W. smithii* tracks climate so closely for several reasons. First, photoperiodism is a physiological adaptation concerned mainly with the timing of seasonal development; second, the low variation in microhabitat offered by the leaves of *Sarracenia purpurea* over a wide geographic range permits a more direct expression of this adaptation. Third, critical photoperiod in *W. smithii* is a continuous variable over the range considered, rather than a discontinuous variable as in some other insects^{21,22}.

To account for geographic differences in the timing of

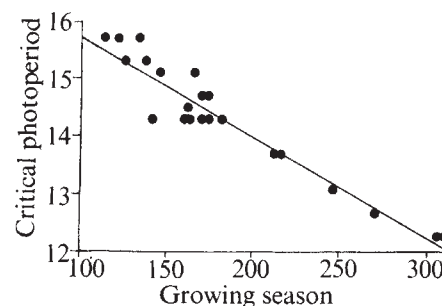


Fig. 2 Correlation of critical photoperiod with growing season. Freeze-free days for each locality were calculated from a least-squares regression equation relating number of freeze-free days of 26 cities in eastern North America to latitude and altitude. $r = 0.96$; $P < 0.001$.

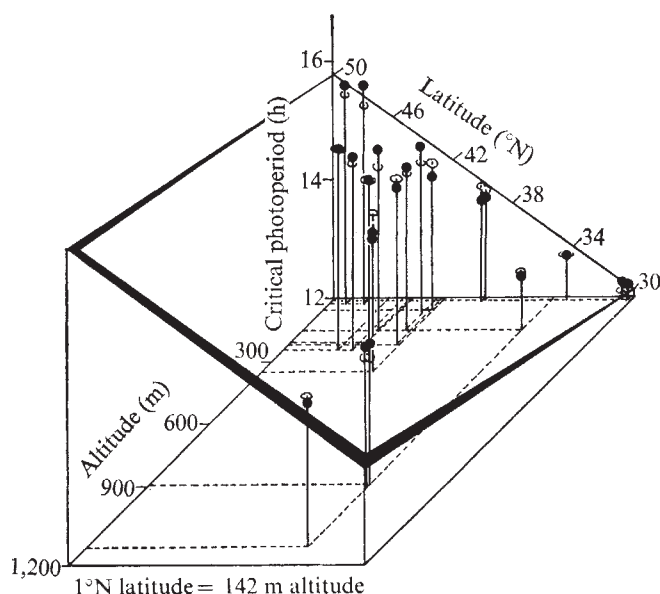
phenological events, including seeding and harvest dates of winter wheat and insect emergence, Hopkins proposed a bioclimatic law²³ equating a 1° increase in latitude with a rise in altitude of 122 m, or somewhat less than my estimate of 142 m per °N. My results are not grounds for modifying Hopkins' law and its prediction of phenological events, but rather point to the departure from this law by the photoperiodic cues which mediate seasonal development. The disparity between Hopkins' law and critical photoperiod in *W. smithii* relates to the observation that, for a given date between the spring and autumnal equinox, daylengths are longer in the north than in the south²⁴. Consequently, insects on a southern mountain should use a shorter daylength to enter diapause on a given calendar date than will those in a population further north which enters diapause on the same date. One would then have to go higher up the mountain than predicted from the bioclimatic law to find a locality where the mosquitoes are using the same critical photoperiod as the northern population. Hopkins' bioclimatic law in temperate and north-temperate latitudes may thus be expected to underestimate the effect of altitude on photoperiodism.

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Fig. 1 Relationship between critical photoperiod, altitude and latitude. Circles on the regression plane indicate where the projections of the data points cross that plane. $r = 0.98$; $P < 0.001$.



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The dispersal of young post-larval bivalve molluscs by byssus threads

New observations, both in the marine environment and in the laboratory, indicate that the byssus in young post-larval bivalves has a hitherto unsuspected function—that of dispersal. The method of transport is analogous to the gossamer flight of young spiders. In the laboratory, small scale currents will induce the secretion of what seems to be a single long thread which, by increasing the viscous drag exerted on the young bivalves, enables them to be carried along on relatively small currents. The threads, which stain readily in Alcian blue (a stain selective for acid mucopolysaccharides) are typically long compared with the length scale of the animal itself: for instance an *Abra* 1 mm long can produce a 2–4- μ m diameter thread of length greater than 3 cm. This mechanism, which we term byssus drifting, is functional from the late pediveliger stage up to at least 2.5 mm size in some species, and it has been found to be present in the 20 species tested so far and listed in Table 1 (a functional byssus has not been reported previously¹ in post-larval bivalves of those genera marked with an asterisk).

The increase in drag produced by the byssus in the process of drifting was investigated experimentally in a simple apparatus which consisted essentially of a vertical glass tube enclosed at either end by fine-mesh netting and into which the animals could be introduced and confined. The upward flow of seawater through the tube was adjusted until a critical current speed, U , was obtained at which an animal could be suspended motionless relative to the tube. Where possible, three sets of readings of U were taken for each animal in each of the following states of activity: (1) live, inactive; that is immediately after the animal has been introduced into the tube and is still closed as a result of being handled, and therefore has not secreted threads into the water, (2) active; that is byssus drifting, and (3) dead (killed in 1% formalin in seawater).

While byssus drifting, an animal hangs from the thread with the ventral shell margins uppermost and held horizontally. The valves are generally only slightly open and the heel of the foot is brought close to the gape. Frequently an animal was observed to detach itself from its thread and fall freely through the tube. In some cases secretion of a new thread would check the descent or even produce upward motion. Sometimes the valves may be opened and shut rapidly several times in succession. At other times the foot is protruded and its anterior part may be wriggled about, or the animal may either swing from side to side (*Mytilus*) or perform what seemed to be digging movements (Tellinacea). The siphons are frequently protruded in the Tellinacea. Contrary to other observations², it was concluded from experimental observations on byssus drifting animals that the protrusion of the foot produces an insignificant increase in drag. Nelson³ reported that post-larval *Mytilus* could remain pelagic by the secretion of gas into the mantle cavity. Gas bubbles have occasionally been seen in the mantle cavity of *Mytilus* during our experiments, but these are possibly caused by transferring the animals from seawater at a temperature of 9 °C to seawater of up to 20 °C in the apparatus.

It is usual⁴ in hydrodynamics to express the effect of fluid drag on a moving body in terms of a dimensionless drag coefficient, C_D , defined as the ratio of the drag force per unit area to the quantity $\frac{1}{2}\rho U^2$, where ρ is the density of the fluid and U is its velocity relative to the body. By the nature of the terminal velocity measurements made in this study, the drag force on the body is simply equal to its weight in water. Assuming uniformity of shape within a given species, the drag coefficient for a body of

Table 1 Species in which byssus drifting has been found to occur

Subclass Protobranchia	
Superfamily Nuculacea	<i>Nucula tenuis</i>
Subclass Lamellibranchia	
Superfamily Mytilacea	<i>Mytilus edulis</i> , <i>Modiolus</i> sp.
	<i>Musculus marmoratus</i>
	<i>Heteranomia squamula</i>
Superfamily Anomiacea	<i>Turtonia minuta</i>
Superfamily Cyamiacea	<i>Montacuta ferruginosa</i> , <i>M. substriata</i>
Superfamily Erycinacea	<i>Cardium echinatum</i>
Superfamily Cardiacea	<i>Venerupis pullastra</i>
Superfamily Veneracea	<i>Mactra corallina</i> *, <i>Spisula</i> * sp.
Superfamily Mactracea	<i>Tellina tenuis</i> *, <i>Gari fervensis</i> *,
Superfamily Tellinacea	<i>Abra alba</i> *, <i>Donax vittatus</i> *
	<i>Cultellus pellucidus</i> , <i>Ensis</i> * sp.
Superfamily Solenacea	<i>Hiatella arctica</i> , <i>H. gallicana</i>
Superfamily Saxicavacea	<i>Mya truncata</i> , <i>Corbula gibba</i>
Superfamily Myacea	

Byssus drifting has been found to occur in all the 22 bivalve species tested so far.

*Those species or genera in which a functional byssus has not been reported previously.

volume, V , surface area, A , and density ρ_b can therefore be written as

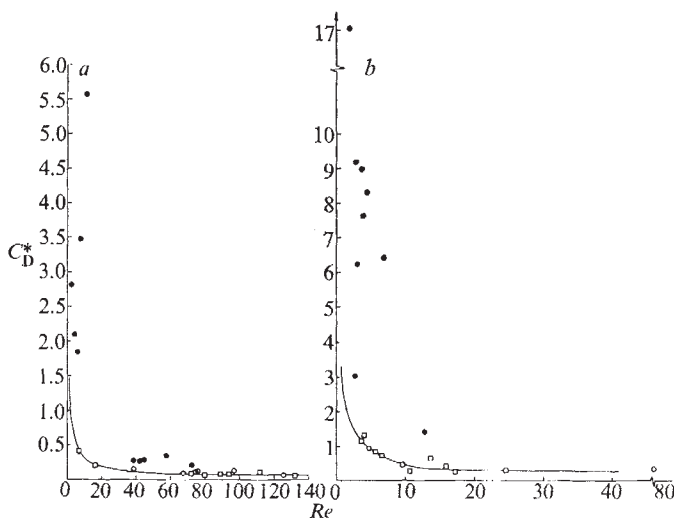
$$C_D = (2g(\rho_b - \rho)V)/A\rho U^2 = kC_D^*$$

where k is a constant determined by the acceleration due to gravity, the relative density of the body, and some geometrical factor directly proportional to its ratio of volume to area. C_D^* is a modified drag parameter equal to a/U^2 , where a is a typical linear dimension of the shell and the constant, k , takes a different value for each species.

Figure 1a and b shows values of the modified drag parameter C_D^* , plotted in the conventional manner as a function of the Reynolds number, Re ($=aU/\nu$, where ν is the kinematic viscosity of the fluid), for the species *Mytilus edulis* and *Abra alba*, respectively. Both graphs show that, in terms of the drag experienced by the bivalves, the dead and inactive samples behave in an almost identical manner, and the form of their behaviour is in excellent agreement with the classical experimental data^{4,5} on the flow past a rigid body, in this Reynolds number range. Small individual departures from the classical curves can be ascribed to small amounts of debris attached to their shells.

Active animals, on the other hand, behave quite differently. This is illustrated by the large random departures from the

Fig. 1 Plot of modified drag coefficient, C_D^* , against Reynolds number, Re , for ●, live active; ○, live inactive; and □, dead samples of *Mytilus edulis* (a) and *Abra alba* (b). The solid curve represents a best fit to the data of the classical experimental results^{4,5} on the flow past a sphere in this Reynolds number range.



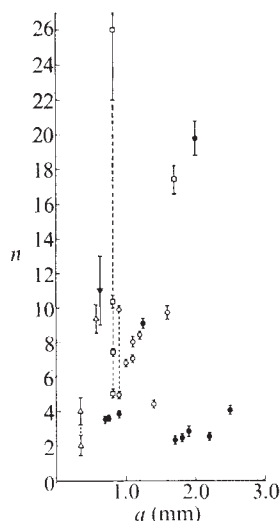


Fig. 2 Measured fractional increase in drag, n , plotted against shell length, a , for ●, *Mytilus edulis*; ○, *Abra alba*; □, *Gari*; △, *Montacuta*; and ▼, *Nucula*.

'dead' curves, indicating the presence of large drag forces which are not due to the shape of their shells alone but must be provided by some other mechanism. Figure 2 shows the relative increase in drag, n , defined as

$$n = (C_D^*)_{\text{active}} / (C_D^*)_{\text{dead}}$$

for active individuals of several species, plotted against shell length, a . It is seen that the drag increase is rarely less than a factor of three, while some adept 'drifters' acquire an increase of 20–30 times the value they experience when inactive. The large errors associated with some values of n are due to the relatively small number of observations in certain size ranges of these species. Hatched lines joining some points show the drag characteristics of the same animals can vary with time, corresponding to various stages of activity.

The experiments certainly indicate that byssus drifting constitutes an effective means of transport for young post-larval bivalves and that the upward components of ocean currents which are required to suspend active individuals are typically only 1 cm s^{-1} . The importance of this mechanism can hardly be doubted and the fact that it seems to be of widespread occurrence in bivalves supports that view. It is almost certainly the mechanism by which post-larval bivalves enter on a second pelagic or migratory phase reported for a few littoral bivalve species^{2,6}, the best documented case being that of *M. edulis*². Preliminary field observations indicate that a prolongation of the larval pelagic phase by a post-larval "bysso-pelagic" phase may be a regular feature of the life history of several sublittoral bivalve species. *A. alba* up to 2 mm in length, and *Montacuta ferruginosa* and *M. substriata* up to 0.8 mm in length, together with post-larvae of several other species, have been taken in considerable numbers in plankton samples. At these sizes the ages of the above species may be from several months to over a year⁷, indicating the possible duration of the "bysso-pelagic" phase. Further investigations into the occurrence and nature of byssus drifting are presently in progress.

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Nematocyst discharge and the effects of antibodies on feeding in hydra

WHILE attempting to isolate hydra morphogens by column chromatography of tissue homogenates, I decided that a bacteria-free culture would be advantageous. Having established that a final concentration of $100 \mu\text{g ml}^{-1}$ penicillin G plus $100 \mu\text{g ml}^{-1}$ streptomycin in the normal¹ culture medium M gave a 100-fold reduction in bacterial contamination in 24 h, I found that hydra could not feed in this solution; indeed, they seemed unaware of *Artemia* larvae bumping into them. On transferring the hydra to fresh medium without antibiotic they were immediately able to feed normally.

I then tested three aspects of normal feeding in hydra: the initial capture by discharge of nematocysts on contact; the subsequent contraction of the tentacles bringing the captured prey to the mouth and the opening of the mouth and engulfment of the prey. Nematocyst discharge can be stimulated by electric shock² mechanical plus chemical stimulation³ or brief sonication. For example, a 0.1-s burst from a Dowe Soniprobe into 10 ml of medium M containing 100 hydra gives >80% discharge of nematocysts, as judged by counting stenothelae using a haemocytometer.

This mechanical discharge can be inhibited by first suspending the hydra in a medium of high osmotic pressure as indicated in Table 1. A 10-s sonication with the microtip at the no. 8 setting in 2 M sucrose gives only 5% discharge. Nuclei are also left intact in these conditions, in spite of rupture of all cell walls and homogenisation of cytoplasm⁴. These observations would seem to confirm the 'osmotic hypothesis' for the discharge of nematocysts⁵ with one reservation: 2 M sucrose has an osmotic pressure equivalent to 1 M NaCl, yet even 2 M NaCl is not so efficient at inhibiting discharge. There is, therefore, the possibility of some additional influence in 2 M sucrose, possibly viscosity, aiding the osmotic effect.

Using a membrane filter with pore size $5 \mu\text{m}$, it is possible to remove nematocysts from the homogenates that are produced and thus obtain a preparation that is relatively free from the nematocyst toxin that is a major component of many 'inhibitory' extracts⁶. The tentacle movement and mouth opening, collectively known as the "feeding response" can be stimulated by $5 \times 10^{-4} \text{ M}$ reduced glutathione in medium M (ref. 7). Table 2 shows the effects of various concentrations of the antibiotics on

Table 1 Percentage discharge of nematocysts on sonication in a medium of high osmotic pressure

Solution	Sonication time and power setting	% Discharge
1 M Sucrose	5 s, no. 8	8.5
	10 s, no. 8	22.5
2 M Sucrose	5 s, no. 8	2.0
	10 s, no. 8	5.0
2 M NaCl	5 s, no. 4	> 10
4 M NaCl	5 s, no. 4	2.5
5 M NaCl	5 s, no. 4	< 1.0
40% Methanol	5 s, no. 4	2.0

Table 2 Effect of antibiotics on feeding response and prey capture

Antibiotic concentration ($\mu\text{g ml}^{-1}$)		Feeding response (estimated means of 10 animals each in $5 \times 10^{-6}\text{M}$ reduced glutathione)				Capture of <i>Artemia</i> (4 hydra + 200 <i>Artemia</i> in 4 ml medium)				
		Degree of response		Time of response (min)			Total number caught		Initial capture rate	
Penicillin G	Streptomycin	Tentacles	Mouth	Tentacles waving for	From	To	For	in 5 s	in 5 min	<i>Artemia</i> per hydra per s
100	100	±	—	4.0	1.5	4.0	2.5	0	0	0.0
10	10	++	±	6.0	0.6	4.6	4.0	40	100+	2.0
1	1	++	+	6.0	0.5	5.0	4.5	40	100+	2.0
100	0	+	—	5.0	1.0	2.5	1.5	48	100+	2.4
10	0	++	±	6.0	0.3	4.3	4.0	50	100+	2.5
1	0	++	±	6.0	0.5	4.5	4.0	50	100+	2.5
0	100	±	—	4.0	2.0	4.0	2.0	0	0	0.0
0	10	+	+	5.0	0.5	5.0	4.5	41	100+	2.1
0	1	++	+	6.0	0.75	5.5	4.75	48	100+	2.4
Control 0	0	++	++	6.0	0.25	5.75	5.5	40	100+	2.0

++, Excellent; +, good; ±, fair; —, poor.

the components of normal feeding in *Hydra littoralis* starved for 24 h. The figures given for the timing of tentacle waving and mouth opening are estimates based on the simultaneous observations of 10 hydra. The lack of precise data for individual animals makes an accurate statistical analysis impossible; the trends, however, are clear.

Together, penicillin G and streptomycin at $100 \mu\text{g ml}^{-1}$ reduce both the length of time that tentacles wave and the time the mouth is open. Mouth opening is also delayed compared with the control but the most important effect is on initial capture, indicating a total inhibition of nematocyst discharge. These effects are totally lost or much reduced at lower antibiotic concentrations.

On its own, penicillin G at $100 \mu\text{g ml}^{-1}$ delays and reduces the mouth opening time but has little or no effect on tentacle waving and nematocyst discharge. Streptomycin, on the other hand, at $100 \mu\text{g ml}^{-1}$ affects all the functions tested, in particular totally inhibiting nematocyst discharge. Again the effects are lost or much reduced when the antibiotics are diluted.

It should be noted that $5 \times 10^{-6}\text{M}$ glutathione is apparently a suboptimal concentration for this particular clone of hydra, since Lenhoff⁷ found a mean mouth opening time of 36 min commencing 0.7 min after application of reduced glutathione.

Streptomycin at $100 \mu\text{g ml}^{-1}$ has, however, no inhibitory effect on artificial mechanical nematocyst discharge by sonication. A temporary paralysis of the cnidocil trigger⁸ is therefore indicated, but the exact mode of action is unknown. The effects on the feeding response suggest that the antibiotics may also interfere with cell to cell communication in hydra, either through the 'nerve net' or the direct electrical contacts that exist between cells⁸. The true significance of this effect is, however, in doubt since a rough χ^2 'goodness of fit' comparison between the most extreme results and the controls gives a chance probability of 0.1. Known inhibitors of the feeding response are structurally similar to glutathione, having the same peptide backbone and apparently acting by competition for a receptor site⁷. No more definite hypothesis can be advanced for the effect of streptomycin, but a warning note should be sounded with respect to the routine use of antibiotics in any experimental situation; there may be unforeseen modifications in cell surface recognition sites, sensory structures or intercellular communication equivalent to those described here.

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Excitability of nerve-free hydra

THE simplest nervous systems known are those of coelenterate polyps, of which hydra is one. Nerve cells of the common freshwater hydra occur in a diffuse, two-dimensional nerve net dispersed among the epithelial cells of the outer, ectodermal layer^{1–4}. There are also a few nerve cells in the inner, endodermal layer but these are scattered and do not seem to form a continuous net⁵. Nowhere in a hydra are there nerve cell concentrations or clusters of sufficient complexity to warrant being called ganglia. There have been many behavioural and electrophysiological investigations of hydra^{6–9}, but the function of the nervous system in the control of behaviour is still unclear. In other coelenterates epithelial cells have been shown to be capable of propagating behavioural signals^{10,11}. Several conducting systems coupled with spontaneously active pacemakers have been identified in hydra, but it is not known which of these conducting systems, if any, are neuronal and which result from activity in non-neuronal, epithelial cells. We have investigated the behaviour of *Hydra attenuata* in effectively nerve-free animals and show that such behaviour as remains, is controlled by non-neuronal cells.

It is now possible to create hydra which are virtually free of nerve cells, by treating the animals with colchicine¹². Colchicine causes the endodermal digestive cells to send processes through the mesoglea and engulf ectodermal nerve cells, interstitial cells and nematoblasts, and draw them into the endoderm where they are digested. After two successive treatments, the hydra consist almost solely of epithelial cells and endodermal gland cells, with only a few cells of the other types remaining. Most of these animals are totally nerve free. Because colchicine treatment removes both the nerve cells and their progenitors, the interstitial cells, these hydra and their buds subsequently remain nerve free. The colchicine-treated animals are somewhat smaller than untreated animals and have altered proportions, but possess all body parts and reproduce by budding. Lacking nematocysts, the treated animals cannot capture prey and are fed by forcing newly hatched *Artemia* through the mouth directly into the gut.

Experimental animals were maintained and studied in 'M' solution¹³ supplemented with $50 \mu\text{g ml}^{-1}$ of the antibiotic rifampicin. The hydra were subjected to two 8-h treatments with 0.4% colchicine spaced two weeks apart¹². They were then fed three times weekly for 3 months before the experiments.

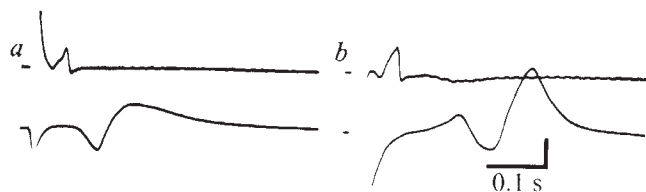


Fig. 1 CPs from a normal (a) and a nerve-free hydra (b). Positive potentials are upward deflections. The animals were stimulated near the base; the upper traces were recorded from a basal disk electrode, the lower traces from an oral electrode. Latency values from the same two animals are plotted in Fig. 2. Vertical calibration: a, 5 mV; b, upper trace 2 mV, lower trace 5 mV.

Control hydra similar in size to the treated hydra were selected from a mass culture: some were kept in M solution and others in M solution containing rifampicin. All animals were starved for 48 h before the experiments.

Electrophysiological studies have previously identified three classes of spontaneous electrical potentials in normal hydra⁹: (1) contraction pulses (CPs), generated by the ectodermal cell layer and associated with symmetrical, longitudinal contraction of the body column; (2) rhythmic potentials (RPs), generated by the endodermal cell layer and associated with contraction of endodermal circular muscles leading to column elongation; and (3) tentacle contraction pulses (TCPs), produced by the tentacles and associated with tentacle contraction. In normal hydra, these potentials occur somewhat sporadically with a mean frequency of the order of 1 per several minutes. Colchicine-treated animals show virtually no spontaneous behaviour, and not a single spontaneous electrical response was recorded in a total of 10 h of recording¹⁴ from five treated animals. The behavioural inertness of colchicine-treated animals is particularly apparent in a time-lapse film, exposed at one frame per 30 s, of a dish containing a normal and a treated animal. In the film (which compresses 33 h of activity into 5 min) the normal animal contracts, elongates and sways from side to side, continuously, whereas the treated animal is motionless except for slight rippling contractions and occasional discharging of gut fluid through the mouth. Passano¹⁵ suggested that a major function of nerve cells in primitive organisms is to initiate spontaneous activity. The absence of spontaneity in colchicine-treated hydra is thus consistent with this view.

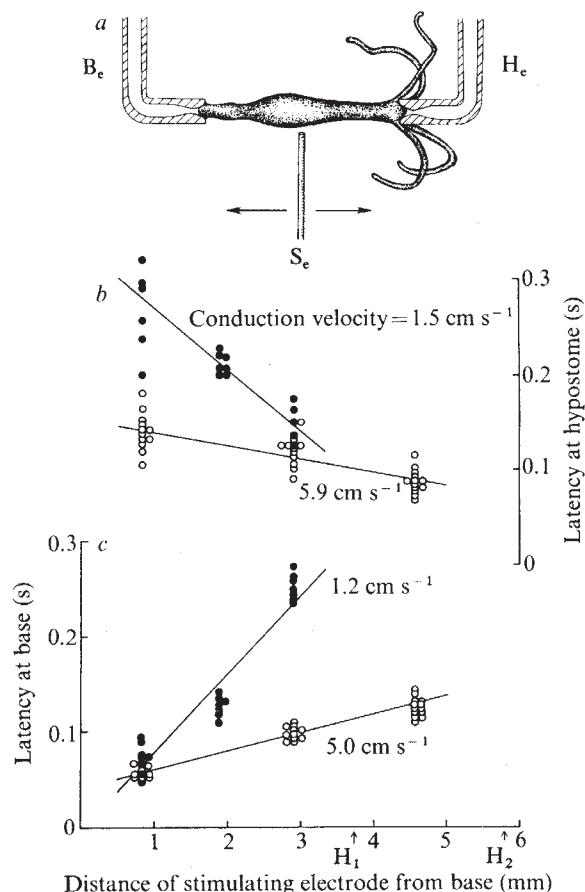
Colchicine-treated animals are relatively unresponsive to external stimuli. Bright light, mild mechanical agitation, and *Artemia* extract all fail to elicit the electrical potentials or behaviour they do in the normal animal⁹. Strong mechanical stimulation, such as pinching with forceps, or electrical stimulation of the column, does however, initiate propagated CPs and column contraction. The CPs of colchicine-treated hydra are of somewhat longer duration than those of normal animals (Fig. 1). The conduction velocity of the CPs was determined by recording them from the basal and oral ends of a hydra while the animal was stimulated at various positions along the column. As a stimulating electrode is moved from the base towards the mouth of an animal, the delay between an applied shock and the appearance of the recorded CP peak increases at the basal electrode and decreases at the oral one. The ratio of change in distance to change in latency gives the conduction velocity⁷ (Fig. 2). Conduction velocity determinations were made from four colchicine-treated hydra, four untreated animals which had been transferred to culture solution containing antibiotic, and four animals grown and tested in normal culture solution. The colchicine-treated animals were the same used to investigate spontaneous activity; one of the latter was too small for conduction velocity measurements. For each animal the column was stimulated sequentially at three points: near the base, near the middle of the column, and near the oral electrode. Usually 10 sets of stimuli were given to each animal. The measured conduction velocity was different in the basal and oral directions, but the direction of preferred conduction varied

from animal to animal. Basal and oral conduction velocities were averaged to give a single value for each animal. The CP conduction velocities of normal animals (5.7 cm s^{-1} , s.e. = 0.4) and antibiotic-treated controls (5.2 cm s^{-1} , s.e. = 0.2) were not significantly different, indicating that the rifampicin did not affect this parameter. These conduction velocities are not significantly different from that earlier reported for *H. littoralis* (5.2 cm s^{-1} , s.e. = 0.3; ref. 7). The CP conduction velocity in colchicine-treated animals (1.8 cm s^{-1} , s.e. = 0.2) was significantly lower than that of untreated animals.

One possible explanation for the reduced conduction velocity in colchicine-treated animals would be that the treatment directly alters the excitability of epithelial cells. In preliminary experiments we have found that the conduction velocity in the column returns to near normal after nerve-free animals are repopulated with interstitial (and thus nerve) cells from a temporary implant of normal tissue¹⁶. This indicates that colchicine does not have long term effects on the epithelial cells and that the depressed conduction velocity is related directly to the absence of nerve cells.

The effectiveness of the colchicine treatment in rendering the animals nerve free was verified by counting cells in macerated preparations of these animals¹⁷. When present, nerve cells are readily detected by this method. In normal animals approximately 5% of the column cells are nerve cells. Samples, each containing ~1,000 cells, were selected from the top, middle and bottom of the column of each treated hydra. The three samples together constituted about half of all the cells in the hydra. No nerve cells were found in four of the five treated animals. In the remaining animal, three nerve cells

Fig. 2 The latency between a stimulus to the column and the appearance of the positive peak of the evoked CP at the basal (c) and oral (b) electrodes. ●, A nerve-free hydra (H_1); ○, a normal hydra (H_2). H_1 and H_2 indicate the total length of each animal. The sketch in a illustrates the electrode placement; B_e and H_e are the basal and hypostomal recording electrodes, and S_e is the moveable stimulating electrode.



were found in samples containing a total of 2,749 non-neuronal cells. All the hydra had about five "big interstitial cells"¹⁷ per 1,000 epithelial cells. Thus, four of the animals were apparently free of nerves and the fifth certainly contained too few nerve cells to form a continuous nerve net.

From these results, we conclude that: (1) nerve cells are necessary for normal spontaneous behaviour in hydra; (2) epithelial cells of hydra are excitable, and can conduct electrical signals which elicit column contraction; and (3) conduction velocity in the column is reduced in the absence of nerve cells. These experiments provide the first direct demonstration of non-neuronal conduction in hydra.

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Directional hearing in crickets

CRICKETS have auditory organs at the proximal ends of the fore-tibiae; the binaural sensory input enables orientation to a sound source and in response to sound, crickets turn towards the more intensely stimulated of their two ears¹. Airborne vibrations activate the auditory tympana of the ears, and are communicated to sensory scolopidia attached to the outer surface of the leg trachea, which forms an air space behind the tympanum^{2,3}. For directionally-sensitive hearing, the responses of the two ears should differ when the direction of incident sound forms an angle with the insect's body axis. Directional hearing in crickets has previously been attributed to shielding of the contralateral ear by the insect's body⁴. We believe that shielding is insufficient to account for observed directionality, and propose an alternative theory which predicts the directional sensitivity of the cricket auditory system measured physiologically.

We examined two species of cricket, *Teleogryllus commodus* and a smaller undescribed species we designate WB2 (specimens of which are lodged with the Australian National Insect Collection, Canberra). In anechoic conditions⁵, we recorded auditory sensory thresholds to the sound frequency of the species song from primary fibres in the leg nerve, and from interneurons in the cervical connective. The directional sensitivity measured at each level in the sensory pathway is similar, and we found no influence of contralateral input on directionality. In normal preparations, crickets were mounted, ventral side up, on a thin vertical support which projected into a uniform sound field radiated horizontally. The prothoracic legs were held with the femora horizontal and perpendicular

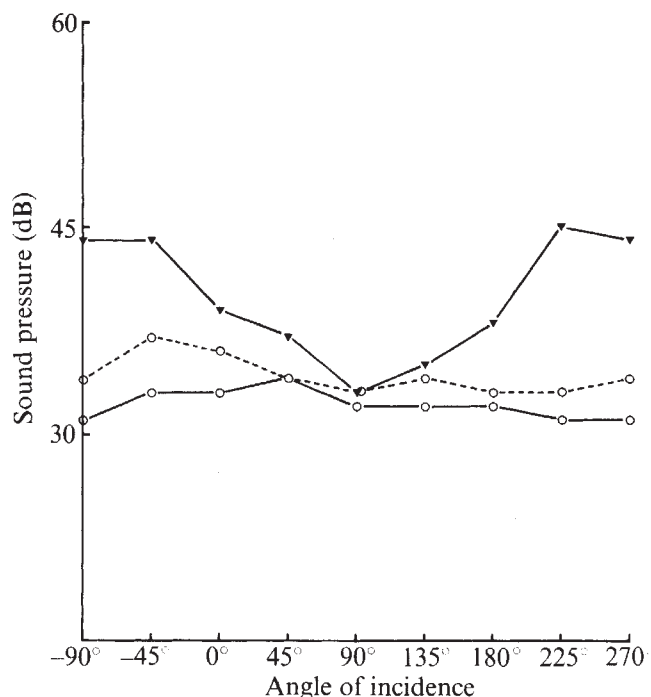


Fig. 1 Auditory thresholds obtained from the cervical connective in species WB2 in response to 4.5-kHz stimuli presented at different angles of incidence. ▽, Intact preparation, note the directional sensitivity; ○, single ear preparations; —, individual ear in a free sound field; —, individual ear asymmetrically shielded with a wax block. The single ear preparations have no directional sensitivity.

to the body axis, and the tibiae projecting vertically. Wings and other legs were removed. In other preparations, the cricket was mounted on its side with one foreleg extended vertically and the other held close to the body and covered with vaseline. In the latter preparations, sound was directed on to one ear only, and the orientation of this ear to the horizontal was near normal, although inverted.

When rotated through 360° in the sound field, normal preparations showed auditory thresholds which differed by 10 to 25 dB for ipsilateral and contralateral incidence of sound. When one ear only was stimulated from different directions, no directional sensitivity was observed. Further, when a wax block approximating in size and shape to the head and prothorax of the cricket was placed next to the single ear, in the position normally occupied by the body, the preparation remained non-directional (Fig. 1). These results indicate that the auditory system is directional only when both ears are stimulated simultaneously, that the individual ear is non-directional, and that shielding of the contralateral ear by the insect's body does not account for the directional response.

In normal preparations, the directional sensitivity is abolished in two ways: (1) by blocking the trachea in either foreleg or in the prothorax and (2) by covering the tympanum of the opposite ear with vaseline. Independently, Zhantiev *et al.*⁶ have demonstrated that directionality of hearing in *Gryllus bimaculatus* is decreased when the contralateral foreleg is removed and the leg trachea blocked. In crickets, therefore, the prothoracic leg tracheal system normally conducts sound from one ear to the other, and the directional response is the result of the interaction of the external and internal sound pressures at each tympanum. If sound is prevented from entering and traversing the internal tracheal pathway in any way, the auditory system is non-directional.

The elements of the prothoracic tracheal system on which directional hearing depends are illustrated in Fig. 2. Simple acoustical considerations explain the basis of the directional

sensitivity. When a plane sound wave is incident at an angle to the body axis, a sound pressure of unit amplitude acting on the outside of the ipsilateral tympanum may be described by $\cos(\omega t)$. The net pressure acting at the ipsilateral tympanum is the difference between the external pressure and the internal pressure transmitted along the trachea from the opposite ear. The phase angle between the external and internal pressures results from the extra distance traversed by sound reaching the inside of the tympanum, namely, the effective distance between the ears, which is a function of the angle of incidence, and the length of the internal tracheal pathway. If it is assumed that there are no other phase changes or amplitude losses, then, at the tympanum

$$P_{\text{net}} = \cos(\omega t) - \cos(\omega t + \frac{l}{\lambda} 2\pi \sin\theta + \frac{L}{\lambda} 2\pi) \quad (1)$$

where l is the external distance between the ears, L is the length of the tracheal pathway, θ is the angle of incidence of sound and λ is the wavelength of sound. In the species we have studied, $l+L$ is nearly half the wavelength of the species' song. (In *T. commodus*, $l+L = 38$ mm, $\lambda/2 = 43$ mm; in species WB2, $l+L = 30$ mm, $\lambda/2 = 38$ mm.) Thus when $\sin\theta = 1$, the phase angle between the external and internal sound pressures at the tympanum approaches π , and the amplitude of P_{net} is a maximum. Conversely, when $\sin\theta = -1$, the phase angle between external and internal sound pressures approaches zero, and the amplitude of P_{net} is a minimum.

Using equation (1) with the appropriate anatomical and song frequency values, we have calculated the relative amplitude of the net sound pressure at the tympanum for values of θ and expressed it in dB. For each species, this calculated net sound

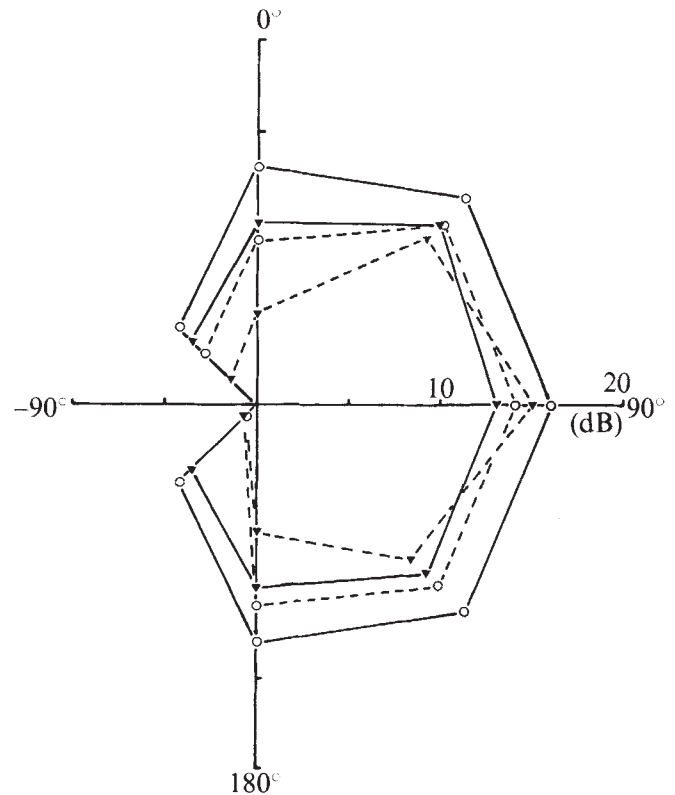
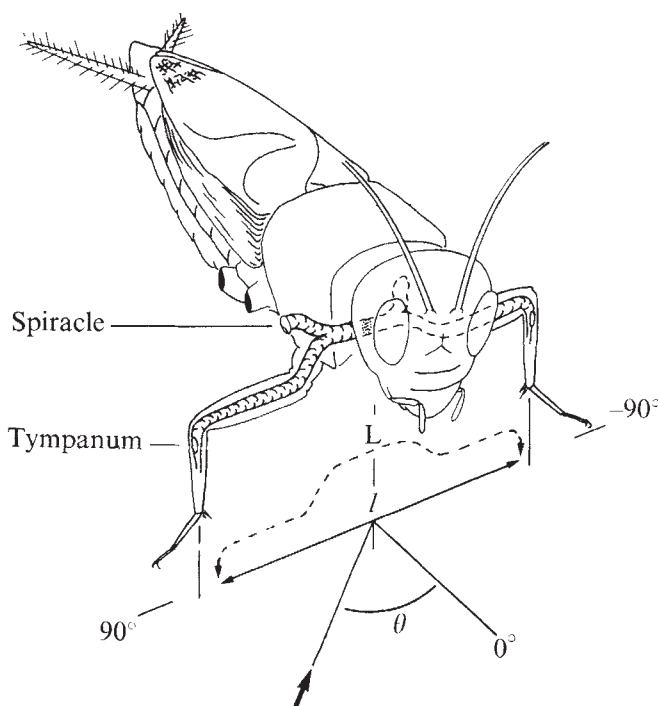


Fig. 3 Predicted net sound pressure at the auditory tympana (—) for values of θ compared with measured hearing sensitivity (---) for *T. commodus* (▼) and species WB2 (○) on polar coordinates.

Fig. 2 Drawing of a cricket showing the apposed leg tracheae which form a sound pathway between the two tibial auditory organs. Sound reaches the exterior and interior of the tympanic membrane along paths of different length. When sound is incident at an angle θ to the body axis, the path to the interior of the ipsilateral tympanum through the tracheae exceeds that to the exterior of the tympanum by the extra distance of the contralateral ear from the source $l \sin\theta$, plus L (the length of the tracheal pathway). Therefore, the net sound pressure at the tympanum is the resultant of the exterior and interior sound pressures, which differ in phase by $2\pi/\lambda (l \sin\theta + L)$, where λ is the wavelength of incident sound.



pressure at the ear for different directions of sound corresponds to the experimental directional sensitivity (Fig. 3).

Transmission of sound along the tracheal pathway is limited with respect to frequency according to the resonance characteristics of the tube. The directional sensitivity of normal preparations applies only to a narrow frequency band near the tuned frequency of the ear, and which corresponds to the frequency of the species' song.

This account of the basis of directional hearing in crickets is consistent with the inability of unilaterally deafened crickets to locate a sound source in phonotaxis trials¹, and with the specificity shown by female crickets in their orientation to signals having different carrier frequencies⁷. The mechanism illustrates the importance of the leg trachea as an integral part of the auditory system. In tettigoniids also, the leg trachea has a crucial, but fundamentally different role in hearing^{6,8}. The cricket derives the directional sensitivity of its ears from the fact that the tracheae of the forelegs are interconnected acoustically at the midline.

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Effect of phloridzin and phloroglucinol on apple shoots

PHENOLIC compounds and their glycosides are widespread in plants. They have uncertain metabolic roles, although they are regarded as metabolic by-products conferring resistance to pathogens^{1,2}. Phloridzin, the 2'-glucoside of phloretic acid, for example, is unique to species of *Malus* and is the major phenolic compound of commercial apple trees (*Malus pumila* Mill), representing 3–7% of the dry weight of leaves as well as occurring in bark and roots³. It is not involved in resistance to apple scab as formerly supposed⁴, but affects the metabolism of many organisms. It inhibits growth of wheat coleoptiles⁵ and tomato roots⁶ but more than doubles photosynthetic rates in *Elodea*^{7,8}. In animals it inhibits the utilisation of carbohydrates⁹, and one of the degradation products^{4,10} of phloridzin, phloroglucinol, acts synergistically with auxin to promote growth of oat mesocotyls¹¹. There have been indications that phloridzin, and phloroglucinol, promote the expansion of apple leaves¹² and the rooting of apple shoots¹³, respectively. This suggests that phloridzin may have important effects on *Malus*. I have now shown that phloridzin promotes the growth of cultured apple shoots. The effect is pronounced and has practical application.

Tips (1–2 cm) of shoots (2–5 cm) of M.7 or M.26 (virus-free) apple rootstocks were cultured as described previously¹⁴ in sterile conditions in test tubes (15 × 2.5 cm), each containing 10 ml culture medium. Shoot tips were kept at 25 °C and received 16 h light daily from Crompton white tubes giving 14 W m⁻² at the surface of each culture tube.

Phloridzin and phloroglucinol (10⁻³ M) were added to a basic medium (medium 1)—Quoirin's medium B (ref. 15) with the vitamins and glycine replaced by the vitamins used by Wetmore and Sorokin¹⁶. For one test with phloridzin, indol-3-yl acetic acid (IAA) was omitted and for others with phloroglucinol, medium 2 was used—as medium 1 but with only 0.5 mg l⁻¹ benzyl amino purine (BA), thiamine hydrochloride (0.4 mg l⁻¹) and inositol (100 mg l⁻¹) in place of the vitamins, and with indol-3-yl butyric acid (1 mg l⁻¹) as ammonium salt (IBA) in place of IAA. All constituents of the media were sterilised by autoclaving at 103 kN m⁻² for 5 min, except for phloridzin, phloroglucinol, and IBA which were passed through Millipore filters (0.45 µm diameter).

Table 1 and Fig. 1 summarise the results and show that the two phenolic compounds markedly stimulated shoot growth. With medium 1 minus IAA, each tip produced a single shoot, and phloridzin increased internode extension, leaf production and expansion. With medium 1 each tip produced between one and three shoots, and phloridzin and phloroglucinol promoted internode extension and increased the number of shoots per tip to between three and six. The shoots originated from terminal

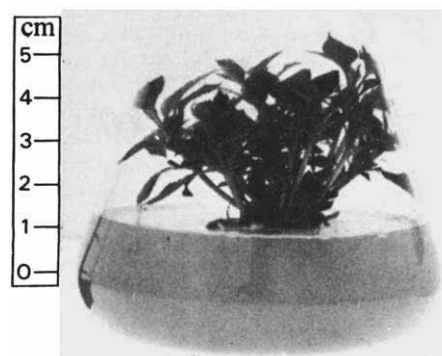


Fig. 1 Effect of phloridzin on M.7 shoots. *a*, Inoculum; *b*, after 5 weeks on medium 1; *c*, after 5 weeks on medium 1 plus 10⁻³ M phloridzin.

and lateral meristems and not *de novo* from callus at the base of cultures (Fig. 1).

Fifteen cultures of M.26 that had produced two shoots each during 4 weeks on medium 1, were transferred singly to 35-ml volumes of medium 2 in 100-ml flasks. This procedure was repeated with medium 2 plus 10⁻³ M phloroglucinol, and in this case 15 cultures were also transferred to 125-ml volumes of medium in 250-ml flasks. After 5 weeks, each culture with 35 or 125-ml medium plus phloroglucinol had produced between 8 and 14 or 20 and 38 shoots, respectively, 2–5 cm long (Fig. 2). With unsupplemented medium 2, however, there were only 3–5 shoots per culture, all less than 2 cm long.

Fig. 2 Effect of phloroglucinol on M.26 shoots. M.26 shoot culture on medium 2 + 10⁻³ M phloroglucinol 5 weeks after initiation from a culture with two shoots.



Table 1 Effect of 10⁻³ M phloridzin (PZ) and phloroglucinol (PG) on apple shoots

Shoots	Medium	No. of replicates	Fresh weight (mg)	Mean growth values*			
				No. of shoots per culture	Shoot length (mm)	No. of leaves ± primordia	Leaf area† (cm ²)
M.7	1 – IAA	15	152	1.0	17	13	1.78
	1 – IAA + PZ	13	257¶	1.0	24§	17‡	2.73¶
M.7	1 + PZ	20	755	2.4	21	14	—
		17	1108¶	3.7¶	29‡	14	—
M.26	1 + PZ	12	715	2.0	17	17	—
		12	1120¶	3.3‡	24‡	16	—
M.7	1 + PG	10	359	1.0	32	19	—
		6	783¶	3.0¶	44¶	20	—

*Culture period 5 weeks except with medium 1 – IAA, where it was 3 weeks.

† Of oldest leaf.

Significantly greater than without PZ or PG ($P = \ddagger 0.05$; §0.01; ¶0.001).

No roots developed in any of the tests described so far, but 100 shoots excised from cultures grown with medium 2 plus phloroglucinol all rooted within 5 weeks when transferred to fresh medium of the same composition but without BA.

These results suggest that phloridzin, possibly through the production of phloroglucinol, is involved in the growth of apple trees. From a practical point of view, the effect on the development of apple shoots has potential for improved propagation of apple trees *in vitro*¹⁸. Methods described recently¹⁷ gave much less shoot growth and rooting than described here with phenolic compounds.

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Malate-switch hypothesis to explain the action of stomata

DURING the past ten years major advances have been made in our understanding of the mechanics of stomata. A most important discovery has been that potassium enters the guard cells in sufficient quantities to account for the decreases in osmotic potential which occur on stomatal opening¹. Association of potassium with stomatal movements has now been reported for at least 22 species². There is doubt, however, about the nature of the anions which accompany K⁺. Chloride has been found to balance about 40% of the K⁺ in *Zea mays*³ and considerably less in *Commelina communis*⁴. Malate could be the balancing anion as it has been found to accumulate in the guard cells on stomatal opening⁵ although it is not clear whether it is synthesised in the guard cells or is transported there from the surrounding cells. Clearly an understanding of the mechanism of the ion transport in and out of the guard cells is necessary as a first step to the unravelling of the stomatal mechanism. An hypothesis is proposed here which attempts to explain the transport aspect of stomatal function. It is based mainly on data obtained from studies with the monocotyledon *C. communis* but should be applicable to other species.

Stomata of *C. communis* have a complex structure with a number of subsidiary cells (Fig. 1). K⁺ and Cl⁻ concentrations in each type of cell have been determined for stomata in both the open and closed states^{4,6}. There seemed to be considerable transport of K⁺ and Cl⁻ between the cells during opening and closure but Cl⁻ never accounted for more than 27% of the K⁺. Table 1 shows data for K, Cl and malate concentrations in the lower epidermis of *C. communis* leaves with open and closed stomata. They show that the total amounts of these ions do not change with stomatal aperture and assuming a valence of 1.5 for malate it approximately balances the K⁺ not accounted for by Cl⁻. It seems reasonable to assume, therefore, that as the malate concentration in the leaf epidermis does not change, malate is not synthesised or broken down to any marked extent

Table 1 Levels of malic acid, K⁺ and Cl⁻ (mol m⁻³) obtained from water extracts of the lower epidermis of the leaf of *Commelina communis*

	Malic acid	K ⁺	Cl ⁻
Stomata open	64.0	147.3	49.2
Stomata closed	61.3	139.9	48.0

Figures are means of five determinations.

but moves with potassium from cell to cell during stomatal activity.

The vacuolar pH of each of the cells of the stomatal complex of *C. communis* changes with stomatal movements. The pH of the guard cell for example, rises from 5.1 to 5.7 when stomata open⁷. Knowing the concentrations of K⁺ and Cl⁻ (refs 4 and 6) and the pH, it is possible to write down the approximate concentrations of mono- and divalent malate in each cell for open and closed stomata (Tables 2 and 3). The data in Tables 2 and 3 are rounded off to make the figures for open and closed stomata symmetrical. The most significant point is that the concentrations of mono-valent malate varies little between the cells both when the stoma is open and when it is closed. On the other hand, there is a steep gradient in the concentrations of divalent malate across the stomatal complex. The gradient is reversed when the stomata open or close.

It is proposed that K⁺ moves from cell to cell with the monovalent malate but that the divalent form of malate is unable to move and is locked in the cell. Cram⁸ has evidence that malate is taken into barley root cells as the singly ionised form rather than as doubly ionised or unionised molecules. It is suggested that stomatal closure brought about by factors such as darkness, high ambient CO₂ and abscisic acid occurs because these factors cause the pH of the guard cells to drop from 5.8 to 5.1. The majority of the malate will switch from the divalent to the monovalent form and a gradient of monovalent malate will develop across the stomatal complex (Table 3). Monovalent malate will thus move across the stomatal complex and eventually reach the epidermal cell whose pH will rise, locking the malate in its vacuole as the divalent form. Equilibrium will be reached when the gradient of monovalent malate is zero and the situation of Table 2 will prevail with the stomata closed.

Stomatal opening would be brought about in the reverse manner. It is proposed that the factors which usually bring about opening such as light, low CO₂, high temperature and circadian rhythms all work by bringing about a rise in the pH of the guard cells. Malate would switch from being predominantly monovalent to being mainly divalent. A gradient in monovalent malate would be set up and K⁺ malate would flow into the guard cells (Table 2). On entering the guard cell the malate would be switched to the divalent form and locked in the cell. The osmotic potential of the guard cell would fall, the turgor pressure rise and the stoma would open. The gradient of monovalent malate would again become zero and a new equilibrium would be attained (Table 3).

It is proposed that the energy for stomatal opening and

Fig. 1 Diagram to show the arrangement of the cells in the stomatal complex of *Commelina communis*.

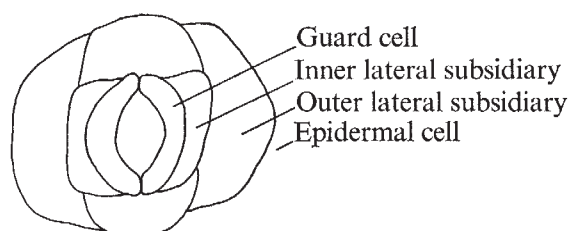


Table 2 pH and concentrations of di- and monovalent malate (mol m^{-3}) in the various cells of the stomatal complex of *C. communis* when the stomata are closed and also about to open

Stomata closed	GC	ILS	OLS	EPID
pH	5.1	5.5	5.7	5.8
Monovalent malate	32	50	45	60
Divalent malate	9	25	53	120
Pre-opening (pH 5.8)				
Malate ⁻	10	50	45	60
Malate ²⁻	20	25	53	120

GC, Guard cell; ILS, inner lateral subsidiary; OLS, outer lateral subsidiary; EPID, epidermal cell.

closing would be expended in bringing about the change in pH of the guard cell from the pK value of malic acid of 5.1 to 5.8 and back again. It is assumed that all the environmental factors which cause stomatal opening and closure work by changing the pH of the guard cell and so operating the malate switch. This would explain why a number of factors can influence stomata by acting independently and also by seeming to act together. For example, before dawn endogenous rhythm could cause a partial rise in guard-cell pH which would be completed by a reduction in ambient CO_2 at the onset of photosynthesis at dawn. This would explain how stomata anticipate dawn by partially opening before the light period. It is not the purpose of this paper to suggest how environmental factors cause the change in guard cell pH.

Table 3 pH and concentrations of di- and monovalent malate (mol m^{-3}) in the various cells of the stomatal complex of *C. communis* with the stomata open and also about to close

Stomata open	GC	ILS	OLS	EPID
pH	5.8	5.7	5.5	5.1
Malate ⁻	60	60	50	32
Malate ²⁻	120	70	25	9
Pre-closing (pH 5.1)				
Malate ⁻	192	60	50	32
Malate ²⁻	53	73	25	9

It follows from this hypothesis that: (1) Opening and closing of stomata should always be preceded by a pH change in the guard cells. (2) The important ion is not K^+ but malate and this would explain why other monovalent cations are effective in bringing about stomatal opening^{9,10}.

Although malate is considered to be the important ion in *C. communis* because of its ability to switch its valence, it is possible that other organic acids may provide the switch anion in some other species. There are, however, few organic acids which have pK values at the pH normally found in plant cells.

The mechanism of transport of the ions between the cells of the stomatal complex is not crucial to the validity of the hypothesis but the gradients envisaged suggest that diffusion could be the most important factor. In this case the active transport found between the cells^{4,6} would be secondarily active. Primary active transport between the cells cannot, however, be ruled out at the present time.

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Plant uptake and leaching of dimethylnitrosamine

THE occurrence of carcinogenic nitrosamines is of considerable interest because they have been found in foods consumed by humans¹ and they can be produced, at least *in vitro*, from agricultural chemicals^{2,3}. Furthermore, the widespread occurrence of potential precursors of nitrosamines—secondary or tertiary amines and inorganic nitrogen compounds that are readily converted to nitrite by microorganisms—led to studies demonstrating that the carcinogens can be formed in soil samples and aquatic environments^{4,5}. A hazardous chemical in soil is not of toxicological significance, however, unless it is volatilised or enters the human food chain by being assimilated by food crops or leached through soil into ground waters ultimately used for drinking. We report here that dimethylnitrosamine (DMNA) can be assimilated by the roots of lettuce and spinach, is translocated to the tops and that it also moves readily through soil.

Lettuce was grown from seed in quartz sand or Williamson silt loam contained in clay pots and incubated at 16 °C in a growth chamber in which the daylength was 16 h. The plants were watered daily with Hoagland's inorganic nutrient solution⁶ at one-quarter strength; the leaves were not wetted during the watering. Spinach was grown from seed in the same nutrient solution, the liquid being aerated constantly and replaced weekly. The spinach plants were maintained at 20 °C under Growlux fluorescent lights timed to provide a 16-h day. After two months, the various pots received either 0.57 μCi of ¹⁴C-DMNA plus the unlabelled nitroso compound to a final concentration of 100 $\mu\text{g ml}^{-1}$, or 0.057 μCi of ¹⁴C-DMNA plus unlabelled nitrosamine to a final concentration of 10 $\mu\text{g ml}^{-1}$. The total amounts of DMNA added were 2.50 and 0.25 mg for the high and low levels of exposure, respectively. After 2 d, the aerial portions of these plants, as well as of plants not exposed to the nitrosamine, were harvested and washed, and 100-g portions homogenised in 150 ml of 6.0 N NaOH. The homogenised slurry was refluxed for 30 min, filtered through glasswool, and steam-distilled, half of the volume being collected as distillate. The distillate was extracted three times with volumes of methylene chloride one-third that of the distillate, and the extract was dried with Na_2SO_4 and concentrated to 2 ml by evaporation in a Kuderna-Danish evaporative concentrator operating at 60 °C. Half of the extract was added to 10 ml of a scintillation cocktail containing 5 g PPO, 0.1 g POPOP, and 1 l of toluene, and the radioactivity was counted on a Beckman model LC-100C liquid scintillation system. A second experiment with lettuce growing in sand was conducted similarly, but the plants were harvested at 4, 9 and 15 d.

The results show that both spinach and lettuce can absorb radioactivity from the growth medium (Table 1), the amount assimilated being proportional to the amount of ¹⁴C-DMNA added to the pots. The absorbed ¹⁴C disappeared with time, in agreement with results on nitrosamine uptake by *Lepidium sativum*⁷. The percentage of radioactivity taken up by lettuce grown in soil was slightly higher than that of plants grown in sand. The quantity of DMNA taken up per unit weight of plant material was, however, severalfold greater for the soil-grown plants, possibly because of the lower yield obtained when the lettuce was grown in the silt loam. It has been reported that cereals can absorb nitrosamines⁸, and the present findings not only extend previous observations, but pertain to two major vegetable crops.

To confirm the identity of the labelled product, 2-month-old lettuce plants were exposed for 2 d to 5.7 μCi of ¹⁴C-DMNA in 25 ml of nutrient solution made to 500 $\mu\text{g ml}^{-1}$

Table 1 Uptake of dimethylnitrosamine by spinach and lettuce

Plant	Growth medium	^{14}C -DMNA supplied (μCi)	Length of exposure (d)	DMNA taken up* (μg per g dry wt)	DMNA taken up by plant* (%)
Lettuce	Sand	0.057	2	1.38	3.20
		0.57	2	14.38	3.25
Lettuce	Soil	0.57	2	106.0	5.06
Spinach	Water	0.057	2	0.54	0.38
		0.57	2	5.60	0.27
Lettuce	Sand	0.57	4	7.04	1.56
			9	1.40	0.21
			15	0.07	0.02

*Each figure represents the average of four replicates.

with the unlabelled nitrosamine. Compounds containing the radioactivity were extracted and concentrated, and then 0.5 ml of the methylene chloride extract was placed on each of two silica gel thin-layer plates (Eastman chromatogram sheets), only one of which was exposed to ultraviolet radiation (240 nm) for 15 min before development. After development in hexane-diethyl ether-methylene chloride (4:3:2), the chromatograms were counted on a strip chart recorder (Actigraph III, Nuclear Chicago), and the location of the radioactivity was compared with that on chromatograms of authentic ^{14}C -DMNA. The radioactivity on the non-irradiated chromatogram had the same R_f value (0.34) as ^{14}C -DMNA, and the radioactivity on the ultraviolet-irradiated chromatogram had the same R_f value (0.03) as ^{14}C -dimethylamine, which is formed by the photolytic

cleavage of DMNA. The yield of dimethylamine was essentially stoichiometric. As a further confirmation of identity, the extract was analysed by gas chromatography using a Varian Aerograph series 1700 gas chromatograph equipped with a flame ionisation detector and a 1.84 m $\times$ 3.2 mm stainless steel column packed with 10% Carbowax 20M on Chromosorb W-HMDS. The column was maintained at 80 °C for 5 min and then programmed to 110 °C at a rate of 2 °C min⁻¹. The retention times of the unknown and authentic DMNA (512 s) were identical.

To determine whether DMNA can leach through soil, columns of Williamson silt loam (pH 5.8, 1.9% organic matter), Lima loam (pH 7.8, 3.8% organic matter), Deerfield sand (pH 5.1, 2.5% organic matter), and Scarborough sand (pH 5.6, 14.8% organic matter) were prepared using 100–200 g of air-dry soil that had been passed through a 2-mm sieve. The columns were moistened with distilled water to bring the soil to field capacity, and a solution containing 100 μg ml⁻¹ of chloride and DMNA (5 μg nitroso nitrogen ml⁻¹) was allowed to pass through the column. Equal fractions (10 ml) were collected, and the fractions analysed for chloride and DMNA content using the methods of Bergmann and Sanik⁹ and Daiber and Preussmann¹⁰, respectively. Nitrite, which gives a positive reaction in the method of DMNA analysis, was determined in all samples by the procedure of Montgomery and Dymock¹¹, and corrections were made for the small amounts present in the first few fractions. The results in Fig. 1 demonstrate that DMNA is not retained by the soils but moves as readily as chloride. Because the soils were open to the atmosphere, some of the added DMNA may have been lost through volatilisation.

The earlier findings that nitrosamines can be formed and are persistent in samples of soil^{4,5,12} and our observation that DMNA can potentially move from soil into food crops and downwards into groundwater suggest that a hazard may exist from a previously unrecognised source of exposure. Investigations on nitrosamine uptake by edible crops and movement through soil are also important in light of the report that the *N*-nitroso derivative of at least one pesticide has been found in a municipal water supply (D. H. Fine, personal communication). Further research is required to determine whether such a possible hazard is in fact a reality.

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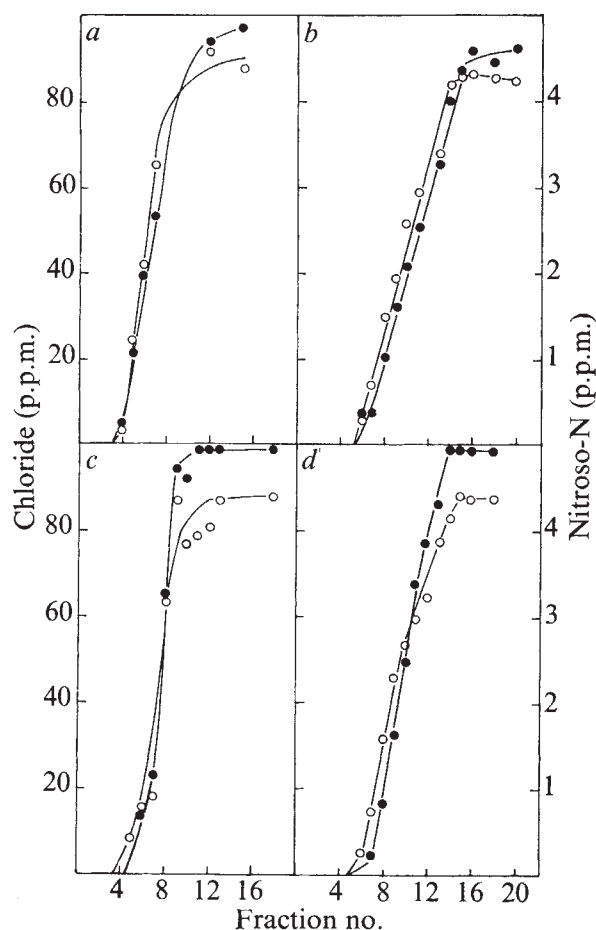
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Fig. 1 Leaching of chloride (●) and dimethylnitrosamine (○) through columns of four soils. a, Deerfield; b, Scarborough; c, Lima; d, Williamson.



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Non-enzymatic photochemical reduction of nitrate in rice seedlings

LIGHT plays an important role in nitrate assimilation in leaves by providing the reductants and by stimulating the synthesis of nitrate and nitrite reductases^{1–3}. Activity of nitrate reductase is considered to be the rate-limiting step in nitrate assimilation and in the production of grains and grain proteins in crop plants^{4,5}. It has been known for some time, however, that the ultraviolet radiation in sunlight photochemically reduces nitrate in aqueous solution to nitrite^{6,7}. Stoy^{8,9} reported that ultraviolet light had a much stronger effect on nitrate assimilation in wheat leaves than light of longer wavelengths. We have observed, in experimental conditions, significant accumulation of nitrite in rice leaves exposed to sunlight, even when nitrate reductase activity was negligible, so we have investigated non-enzymatic photochemical reduction of nitrate in leaves.

The absorption spectrum of 0.2 M KNO₃ measured in a Gilford spectrophotometer showed a trough at 260 nm ($E = 0.3$) and a sharp peak at 302 nm ($E = 1.04$). Negligible absorption was observed at wavelengths 340 nm and above. On the other hand, 0.2 M NaNO₂ revealed a broad absorption band ($E = 1.25$ – 1.75) in the whole range of wavelengths from 260 to 325 nm, after which it showed a decline, with negligible absorption at 400 nm and above.

When 10 mM KNO₃ solution was exposed to bright sunlight of intensity from 7,000 to 10,000 foot candles (70–108 klx), about 10 to 20 nmol NO₂[–] ml^{–1} h^{–1} was produced. This could be compared with the amount of nitrite produced *in vitro* by an

active preparation of nitrate reductase from rice leaves, incubated with 0.34 mM NADH and 10 mM KNO₃, which was found to be 20–30 nmol NO₂[–] ml^{–1} h^{–1}. When 10 mM NaNO₂ was irradiated with sunlight, negligible loss of nitrite was observed.

That the ultraviolet component of solar radiation was responsible for the reduction of nitrate was easily confirmed by using filters, which absorbed that part. Negligible quantities of nitrite were formed when nitrate solution was treated with such filtered sunlight. Exposure of nitrate to ultraviolet light from a mercury lamp was as effective for reduction as direct sunlight.

To detect non-enzymatic accumulation of nitrite in leaves exposed to sunlight, it was necessary to eliminate, or diminish considerably, the activities of both nitrate and nitrite reductases. In order to achieve this, rice seedlings were grown in complete darkness. It is known that synthesis of the two enzymes is retarded in etiolated plants^{2,3}. When these leaves are exposed to sunlight, however, the enzymes are likely to be synthesised as the chloroplasts develop and to prevent this, nitrate was added in the presence of sodium tungstate and chloramphenicol. Tungstate blocks the *in vivo* synthesis of an active nitrate reductase, by forming the enzymatically inactive tungsten analogue by replacement of molybdenum in the enzyme molecule¹⁰. Chloramphenicol, an inhibitor of protein synthesis on 70S ribosomes, inhibits the formation of nitrite reductase, which requires protein synthesis on chloroplastic ribosomes, without affecting the synthesis of nitrate reductase, which takes place on 80S ribosomes in the cytoplasm^{3,11}. Thus, by a combination of tungstate and chloramphenicol treatments, we hoped to diminish the activities of nitrate reductase and nitrite reductase considerably.

Results show that in normal seedlings grown in sunlight with nitrate (Table 1; treatment 1), the activities of the two enzymes were quite high. Free nitrite was not detected in the leaves because of its rapid reduction to ammonia in healthy seedlings. The seedlings in which the activities of the two enzymes were blocked (treatment 3) progressively accumulated nitrite for up to 4 h in leaves exposed to sunlight. The same result was brought about by ultraviolet light from a mercury lamp; but detached leaves kept in the dark did not accumulate nitrite. When the seedlings were treated with chloramphenicol (treat-

Table 1 Effect of sunlight on the accumulation of nitrite in leaves of rice seedlings

Treatment number	Conditions of growth of seedlings Light regime	Additions to nutrient solution	Period of exposure of detached leaves to sunlight (h)	Nitrate reductase (μmol NO ₂ [–] per 20 min per g tissue)		Nitrite reductase (μmol NO ₂ [–] reduced per 20 min per g tissue)	Nitrate (μmol per g tissue)	Nitrite (μmol per g tissue)
				<i>In vitro</i>	<i>In vivo</i>			
1	Sunlight	KNO ₃	4	1.92	0.182	13.4	12.1	Nil
2	Sunlight	KNO ₃ + chloramphenicol	4	1.08	0.096	1.2	11.4	0.220
3	Dark	KNO ₃ + tungstate + chloramphenicol	0					0.012
			1					0.027
			2					0.044
			3					0.062
			4	0.04	Nil	1.0	12.2	0.083
		(Detached leaves kept in the dark)	4					0.012
		(Detached leaves exposed to ultraviolet light from a mercury lamp)	1					0.041

Seedlings of rice (*Oryza sativa*), variety Taichung Native-1 were raised in well washed quartz sand with Hoagland solution lacking nitrate as described earlier³. Seedlings grown in sunlight in treatments 1 and 2 were exposed during the day (10 h) and kept in dark during the night. In treatment 1, 15 mM KNO₃ was added on the 14th day. In treatment 2, 15 mM KNO₃ and 3 mg ml^{–1} chloramphenicol were added on day 14 and seedlings kept in the dark for 2 d. In treatment 3, on day 14, 15 mM KNO₃ with 5 mM sodium tungstate and 3 mg ml^{–1} chloramphenicol were added to the nutrient solution and also sprayed on the etiolated plants and seedlings kept in the dark for 2 more days. Leaves from 15-d-old seedlings from the three treatments were detached from plants and immersed in 15 mM KNO₃ solution so that the bottom 1 cm dipped into the solution and the remaining portion was exposed to sunlight. The nitrate solution was kept in flasks covered with a black paper, so it was not exposed to sunlight and photochemical reduction of nitrate in the flasks was avoided. The protruding leaves alone were exposed to light for different intervals as indicated in the table. At the end of this period, leaf extracts were prepared and *in vitro* activities of nitrate reductase and nitrite reductase were assayed as described previously, using NADH and reduced methyl viologen as electron donors, respectively³. *In vivo* nitrate reductase activity in the leaf disks was assayed by the method of Jaworski¹². Nitrite content in the leaf extracts as well as in the enzyme assays, was determined by the standard Greiss-Ilosvay colorimetric method. Nitrate content in suitably diluted leaf extracts was determined after its quantitative enzymic reduction to nitrite by an active dialysed nitrate reductase obtained from rice leaves.

ment 2), the activity of nitrite reductase was negligible, whereas nitrate reductase was synthesised during a 4-h exposure of green detached leaves to sunlight. In these conditions, large quantities of nitrite accumulated. The amount of nitrite accumulated *in situ* was about three times that formed by the assumed photochemical reduction observed in treatment 3.

Activity *in vivo* of nitrate reductase in leaf disks was less than 10% of the *in vitro* activity in leaf extracts, assayed in the presence of excess NADH. Similar results indicating a comparatively low *in vivo* activity of nitrate reductase have been reported^{13,14}. Moreover, it is uncertain whether either the *in vivo* or *in vitro* assay is a true reflection of the *in situ* nitrate assimilation. The estimated input of reduced nitrogen based on *in vivo* enzyme assay is known to exceed substantially the actual accumulation of reduced nitrogen by the plant *in situ*⁵. Our results show that photochemical reduction of nitrate could be as large as 30% of the *in situ* reduction.

In plants exposed to very bright sunlight, as in tropical countries, photochemical reduction of nitrate could thus be significant. Activity of nitrate reductase alone may not, therefore, be a factor limiting the rate of nitrate assimilation.

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Immunopathology of schistosomiasis in athymic mice

EOSINOPHILIA—tissue eosinophil infiltrates—granuloma and IgE formation are associated with helminth infections. Eosinophils phagocytose antigen-antibody complexes, neutralise histamine and serotonin, mediate inflammatory reactions and modify delayed hypersensitivity¹. The tissue-invasive stage of some parasitic infections provides suitable antigen and stimulates the production of IgE, which in turn induce eosinophilotaxis. We have investigated the need of T-cell participation in the eosinophil response, IgE formation and granuloma formation in schistosomiasis in congenitally athymic mice which have profound deficiency in T-cell dependent immune response. We looked in particular at eggs as they are important in the pathogenesis of schistosomiasis. They are trapped in tissues and induce chronic granulomatous inflammation with numerous eosinophils. Warren *et al.*²⁻⁶ reported that egg granuloma is a cell-mediated hypersensitivity reaction and it is significantly altered in immunosuppressed mice.

Sixteen homozygous athymic nude (*nu/nu* mice with an outbred Swiss mouse background and 16 normal heterozygous (*nu/+*) littermates 9 weeks old were housed in specific pathogen-free conditions (mouse hepatitis virus). They were infected through tail immersion with 35-40

Schistosoma mansoni (Puerto Rico strain) cercariae freshly released from the intermediate snail host, *Biomphalaria glabrata*. This level of infection results in a chronic murine hepatosplenic disease which closely resembles, pathophysiologically, human chronic schistosomiasis⁷. Animals were killed 52, 58 and 66 d after infection. Although *nu/+* mice had ruffled hair coat and hunched back, *nu/nu* mice showed no remarkable signs of illness. The hepatosplenomegaly and enlargement of mesentery lymph node associated with schistosomiasis was significantly more severe in *nu/+* mice. Microscopic examination of the liver showed that *S. mansoni* eggs were trapped in the small intrahepatic blood vessels and granuloma was formed around them. In the immune-intact normal mice, the egg granuloma was a circumscribed lesion consisting of predominately eosinophils, macrophages, lymphocytes, epithelioid cells, granulation tissue and sometimes giant cells. Reparative fibrosis was distinct. The white necrotic hepatic lesions in athymic mice were smaller than those in control mice. The sizes of egg granulomas, determined by measuring two diameters at right angles to each other with an ocular micrometer 52, 58 and 66 d after infection, are presented in Table 1. The mean sizes of granulomas in athymic mice were significantly smaller than those in heterozygous mice at $P < 0.001$ as calculated by Student's *t* test. Granulomas decreased in size during the course of infection. No tissue eosinophils were found in the egg granuloma in the athymic mice, whereas neutrophils, macrophages, lymphocytes, epithelioid cells and pigments were usually present. We did not see giant cells in granulomas. Occasionally only a few or no inflammatory cells were found around the eggs. Granulation tissue or collagen formation was observed in some egg granuloma lesions, although they were not as well established as those

Fig. 1 The lymphocyte transformation of splenic cells of athymic (*nu/nu*) and heterozygous (*nu/+*) mice 52 d after infection with 35-40 *S. mansoni* cercariae. The blastogenic activities stimulated with 20 µg of PHA, 40 µg of LPS and 12 µg of adult *S. mansoni* antigen, are expressed as the stimulation index by comparing the thymidine uptake by infected splenic cells with the thymidine uptake by control (unsensitised) splenic cells. The spleen cell suspension (2×10^5) was added to each well of microtrays and cultured in triplicate. The soluble adult antigen was prepared by homogenised lyophilised worms in RPMI 1640 medium, centrifuged at 2,500 r.p.m. for 30 min. The supernatant was sterilised by passing through a 0.45-µm filter and used as antigen. After 72 h of incubation at 37 °C, 2 µCi of ³H-thymidine were introduced to each well; incubated for another 16 h, cells were collected, extracted and counted in a scintillation counter.

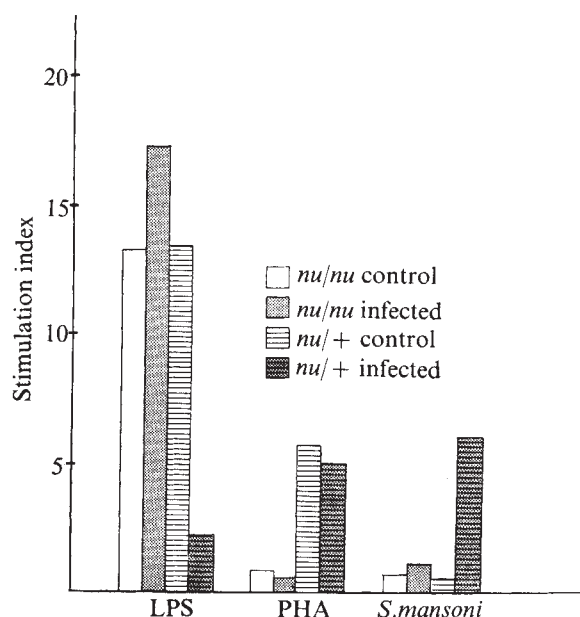


Table 1 Comparison of egg granulomas in athymic and heterozygous mice infected with 35–40 *S. mansoni* cercariae

Time	Day 52		Day 58		Day 66	
Genotype	<i>nu/nu</i>	<i>nu/+</i>	<i>nu/nu</i>	<i>nu/+</i>	<i>nu/nu</i>	<i>nu/+</i>
No. mice studied	4	4	6	6	6	6
No. granulomas measured	52	41	70	53	64	46
Granuloma size (μm)						
range ($L \times W$)*	57–660	76–780	57–456	114–576	49–313	161–523
$\times 47$ –340		$\times 48$ –540	$\times 48$ –342	$\times 113$ –475	$\times 47$ –247	$\times 85$ –486
mean ($L \times W$)†	254 $\times$ 192	479 $\times$ 341	214 $\times$ 141	379 $\times$ 291	161 $\times$ 104	381 $\times$ 293
s.d. ($L \times W$)	111 $\times$ 83	143 $\times$ 115	97 $\times$ 67	112 $\times$ 91	57 $\times$ 38	90 $\times$ 80
P	<0.001		<0.001		<0.001	
% Eggs without cell response‡	9	2	6	0	6	0

* $L \times W$, range of length (L) by range of width (W).

†Mean of length by mean of width.

‡Measurement included in granuloma size.

in normal mice. Small granulomas with few eosinophils were observed occasionally in the intestinal wall of the athymic mice.

Blood eosinophilia was studied in seven athymic and 10 normal mice before and after infection at weekly intervals. Tail blood smears were made at about 1400 and were stained by the Jenner–Giemsa method. A total of 300 leukocytes was examined per slide. Circulating eosinophils were observed in infected athymic mice (2–4%) although their levels were significantly lower ($P < 0.001$) than those in infected heterozygotes (10–12%) after 7 weeks infection. Evidently the eosinophilopoiesis in athymic mice was not impaired. The role and specific function of eosinophils in the granuloma has not been established. According to Walls *et al.*⁸ the destruction of liver tissue by eggs should stimulate the local eosinophil response. This apparently is not true in infected nude mice. The migration of tissue eosinophils to the affected sites seems to be dependent on T cells or other thymus factors which are absent in athymic mice. Eosinophils are known to ingest antibody–antigen complex. The complex may not be formed in nude mice because of the lack of the functional T cell required to produce anti-parasitic antibodies.

The lymphocyte responsiveness of athymic mice and heterozygotes to specific adult *S. mansoni* antigens and to nonspecific mitogens, phytohaemagglutinin (PHA) and *Salmonella typhimurium* lipopolysaccharide (LPS) was determined by measuring the incorporation of ³H-thymidine into DNA. The blastogenesis of spleen cells of infected and non-infected athymic mice and heterozygotes is shown in Fig. 1. The B cells of both non-infected and infected nude mice, as well as the non-infected heterozygotes responded

strongly to LPS, while the B-cell function of infected *nu/+* mice was greatly depressed. T cells of non-infected and infected *nu/+* mice reacted vigorously to PHA stimulation in contrast to those of nude mice. In the response to adult *S. mansoni* antigen, infected *nu/+* mice reacted strongly, whereas the nude mice (infected and non-infected) and control *nu/+* mice spleen cells were not stimulated by the specific antigen. This evidence indicates that the lymphocyte responsiveness to *S. mansoni* is dependent on T cells or thymus factor.

The relationship between functional T cells and reagenic antibodies (IgE and IgG₁) was studied in nude and *nu/+* mice infected with 100 *S. mansoni* cercariae. We found that T-cell deficient nude mice were unable to produce detectable levels of IgE and IgG₁ antibodies whereas the *nu/+* mice produce both long term skin-sensitising IgE antibody and short term skin-sensitising IgG₁ antibody as early as 49 d after infection (Table 2). Both IgE and IgG₁ persisted in the blood for more than 10 weeks after infection. Evidently, the IgE and IgG₁ formation in response to *S. mansoni* antigens may require the participation of T cells. We have not observed significant quantitative differences in levels of both IgE and IgG₁ antibodies in *nu/+* mice responding to soluble adult or egg antigen. Mota *et al.*⁹ were unable to detect reagenic antibody in ICR mice infected with 150 *S. mansoni* cercariae by passive cutaneous anaphylaxis (PCA) in mice. Our finding, however, confirms the recent report¹⁰ that both IgE and IgG₁ were produced in CBA/J mice with chronic schistosomiasis. The lack of reagenic antibodies in nude mice may contribute to the absence of the local tissue eosinophil reaction¹.

Buchanan *et al.*¹¹ failed to induce egg granulomas in the liver and intestines of experimentally T-cell-depleted mice. Our study, however, has demonstrated that congenitally athymic mice can form granulomas around *S. mansoni* eggs. The lymphocytes in granulomas of athymic mice might be B-lymphocyte-related cells that respond to certain T-cell independent antigens. Therefore the egg granulomas may be mediated partly by B cells, if not solely by T cells. This remains to be studied. We did not see giant cells in the granulomas of athymic mice, although it is not uncommon in normal mice. The observation suggests the association of giant cell formation with thymus factor¹².

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Table 2 Titre of reagenic antibodies in sera of *nu/nu* and *nu/+* mice infected with 100 *S. mansoni* cercariae determined by the PCA reactivity in rats or nude mice

Genotype	Time after infection							
	Day 49		Day 53		Day 58		Day 72	
	IgE	IgG ₁	IgE	IgG ₁	IgE	IgG ₁	IgE	IgG ₁
<i>nu/+</i>	40	40	10	20	40	30	10	40
<i>nu/nu</i>	0	0	0	0	0	0	0	0

The presence of IgE in pooled sera was measured by the PCA test in rats (adult male Sprague–Dawley) with a latent period of 24 h and in female nude mice with 72 h of sensitisation. The IgG₁ antibody was determined by PCA reactions in nude mice with a latency of 3 h. A sample of 0.05 ml of diluted sera was injected intradermally into each skin site. The antigens for intravenous challenge were soluble crude extracts from adult *S. mansoni* worms or eggs and were adjusted to 0.8 mg of protein per ml of solution. The volume was 1 ml per rat and 0.2 ml per mouse in mixing with equal amounts of 1% potamine sky blue dye solution. The size and bluing intensity of PCA reactions on the underside of skin were read 20–30 min after antigen challenge through the tail vein. The highest dilution of sera giving a reaction, 5 mm greater than normal serum control in at least half of PCA recipients, was regarded as the titre.

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Growth of *Mycobacterium leprae* and *M. marinum* in congenitally athymic (nude) mice

THE failure to grow the leprosy bacillus, *Mycobacterium leprae*, *in vitro* means that there is a requirement for a highly susceptible experimental animal model. The only animal confirmed so far as naturally highly susceptible is the nine-banded armadillo, of which at least some specimens develop, after inoculation, a disseminated disease which resembles human lepromatous leprosy¹. The inoculation of *M. leprae* into the footpads of mice results in a localised, self-limiting infection, with a maximum bacillary yield of about $10^{6.5}$ organisms per footpad². Suppression of the immune response of the mouse, by thymectomy and irradiation, results in enhancement of the footpad infection, and dissemination of *M. leprae* bacilli to internal organs and peripheral tissue such as ears, nose and tail³. This ability to enhance infection by nonspecific depression of T-cell function suggests that the congenitally athymic, or nude, mouse might provide a suitable, naturally susceptible host for *M. leprae*. In spite of the severe impairment of the cellular immune response of nude mice⁴⁻⁷, the only report of the growth of *M. leprae* in such mice concluded that there was no significant increase in bacillary proliferation, and no tendency to promote a generalised infection⁸. This conclusion was based, however, on observations made only 6 months after inoculation—an interval that is almost certainly too short for detection of significant enhancement or dissemination.

To investigate further the growth of *M. leprae* in nude mice, we established a small breeding colony based on the mating of a closed, but not inbred group of mice. Heterozygous females (nu/+) were mated with homozygous males (nu/nu) to give a progeny half homozygous and half heterozygous. The homozygous mice were used as experimental (nude) mice, and the heterozygous, phenotypically normal littermates were used as controls.

In a preliminary experiment, *Mycobacterium marinum* was used as the infecting agent. Infection of mice with *M. marinum*

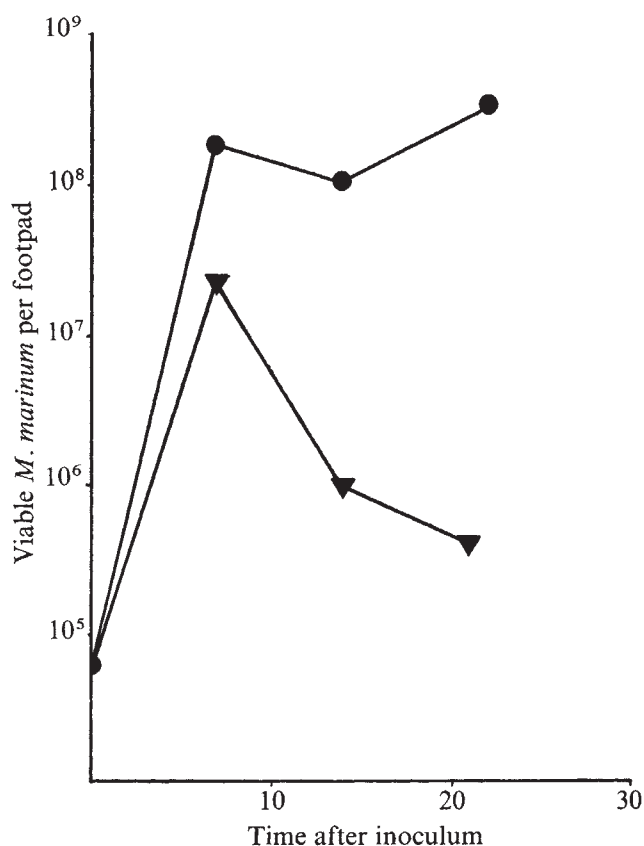


Fig. 1 Growth of *M. marinum* in the footpads of nude mice (●) and control mice (▼). Each sample represents the viable count of bacilli from the pooled tissue of three mice. Footpads were removed and homogenised in Middlebrook-Dubos medium, and viable counts made by the method of Miles and Misra¹³.

and *M. leprae* is analogous⁹; growth of both organisms reaches a maximum of 10^6 – 10^7 bacilli per footpad, followed by killing of the organisms. But, the fact that *M. marinum* grows much more rapidly than *M. leprae* and is cultivable *in vitro* makes it a convenient model for the study of the *M. leprae* infection.

Mice were inoculated with *M. marinum* in the left hind footpad and bacillary growth was monitored as shown in Fig. 1. *M. marinum* grew to a peak of $10^{7.2}$ viable bacilli per footpad by 7 d in control mice, and this was followed by a rapid decline in viable numbers, falling to $10^{5.4}$ at 21 d. In the nude mouse group, the number of organisms had reached $10^{8.2}$ per footpad by 7 d, and although this was followed by a plateau, there was no decline in viable numbers.

At the same time that footpads were collected, liver, lungs, spleen and the contralateral footpads were also removed and examined for the presence of viable bacilli (Table 1). Viable organisms were detectable in the internal organs of nude mice at all collections, whereas at no stage of the infection were viable organisms recoverable from the internal organs of control mice.

To study the growth of *M. leprae* in nude mice, 30 nude and

Table 1 Recovery of viable *M. marinum* from mouse tissue after inoculation in the left hind footpad

Time after infection (d)	Right footpad	Nude mice (viable <i>M. marinum</i> per organ)			
		Left footpad	Liver	Spleen	Lung
7	1.9×10^8	—	1.3×10^4	1.5×10^4	2.4×10^3
14	1.1×10^8	—	4.4×10^3	6.4×10^5	1.6×10^3
21	3.6×10^8	—	7.2×10^6	8.6×10^6	1.4×10^6
Control mice (viable <i>M. marinum</i> per organ)					
7	2.4×10^7	—	—	—	—
14	9.6×10^5	—	—	—	—
21	4.0×10^6	—	—	—	—

Each sample represents the viable count of bacilli from the pooled tissue of three mice. Tissue was removed and homogenised in Middlebrook-Dubos medium, and viable counts were made by the method of Miles and Misra¹³. — Represents $<10^3$ viable *M. marinum* per organ.

60 control mice were inoculated into both hind footpads, with bacilli from a biopsy specimen from an untreated lepromatous leprosy patient. The results are shown in Fig. 2. Because of the poor survival of nude mice, they were not killed for collection, but footpads were removed and bacillary counts were made at their natural death. Collection of *M. leprae* from mouse tissue, and counting of acid-fast bacilli were carried out as described before¹⁰. There was no evidence of an increase in bacillary numbers in any of the mice during the first 4 months after inoculation, but while growth in the control group reached a maximum at about 10^6 bacilli per footpad, counts in nude mice continued to rise. Enhancement of bacillary growth in the two longest surviving nude mice, collected at 266 and 322 d, was highly significant ($P \leq 0.01$), and was accompanied by clearly visible swelling of the footpads.

The longest surviving nude mouse was examined for the presence of acid-fast bacilli in ears, nose, testes, tail, forepaws, liver and spleen (Fig. 3). A few bacilli were detectable in the testes, nose, tail and forepaws, though in the testes, tail and forepaws, the numbers were only just above the level of detection and represent only one or two bacilli observed by the counting technique. Significant levels of bacilli were present in liver and spleen, however, with $10^{5.6}$ and $10^{5.3}$, respectively. The liver and spleen are also heavily infected with leprosy bacilli in the highly susceptible nine-banded armadillo¹¹. No acid-fast bacilli were recoverable from the internal organs or peripheral tissue, other than the inoculation site, in control mice.

Confirmation of the identity of the *M. leprae* bacilli collected from nude mice was made by removal of acid fastness by pre-treatment with pyridine¹², and by subinoculation into the footpads of normal mice—bacillary growth reached a maximum of 10^6 120 d after inoculation, conforming to the classical growth pattern of *M. leprae*.

Thus, in contrast to the findings of Prabhakaran *et al.*, whose observations were made over only 6 months, we found

Fig. 2 Growth of *M. leprae* in the footpads of nude mice. Each point on the control curve (○) represents the mean value of twelve mouse footpad counts, and the shaded area is the 95% upper confidence limit of the control group during the plateau phase. Nude mouse values (●) are the bacillary counts of individual footpads removed at autopsy.

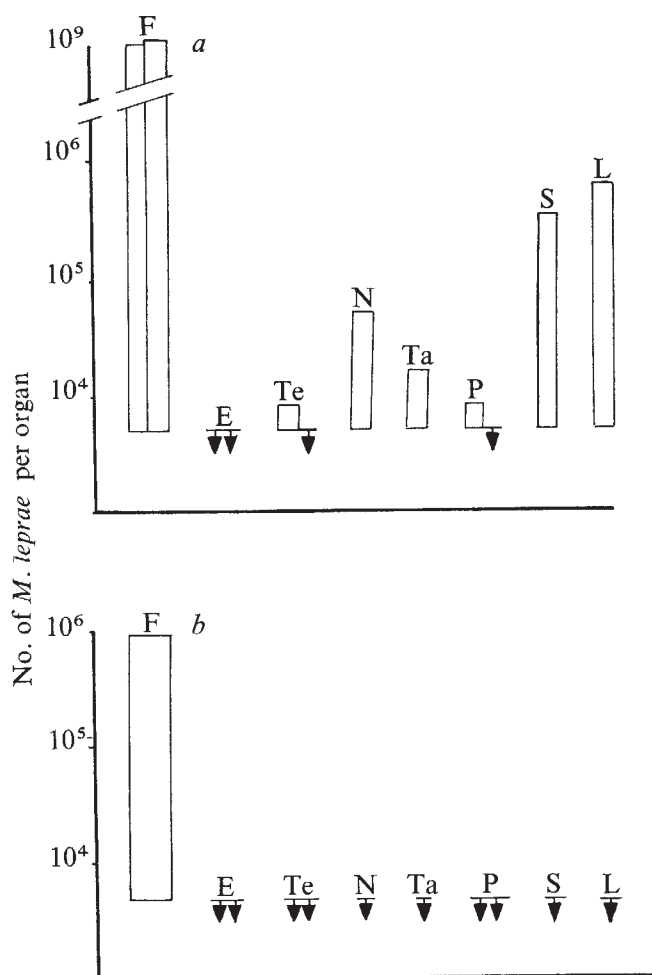
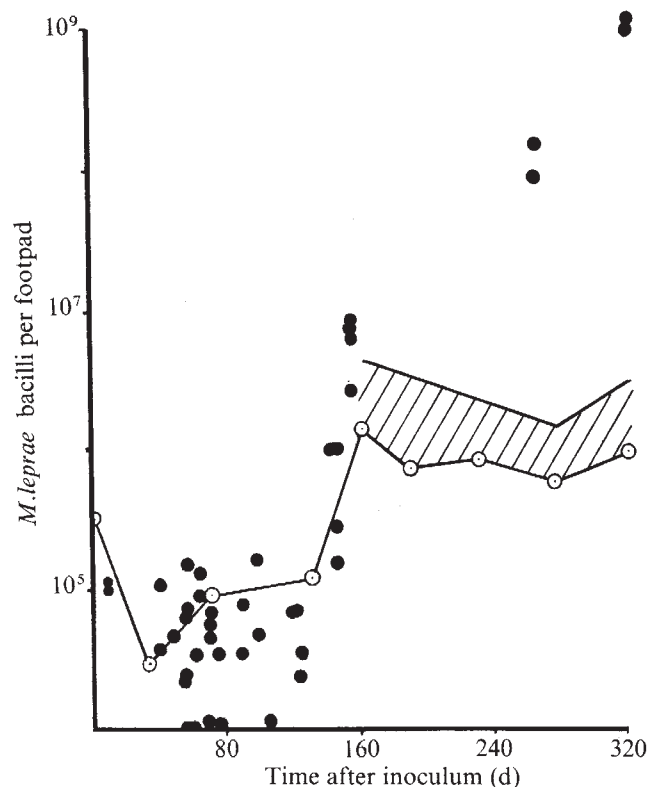


Fig. 3 The recovery of *M. leprae* from the internal organs and peripheral tissue of the longest surviving nude mouse, and from control mice (mean value of six mice), following footpad inoculation. All mice were sampled 322 days after inoculation. F: footpads; E: ears; Te: testes; N: nose; Ta: tail; P: forepaws; S: spleen; L: liver.

that inoculation of *M. leprae* into the footpads of nude mice resulted in a very significantly enhanced infection compared with immunologically normal mice; and the recovery of significant numbers of bacilli from the spleen and liver of the longest surviving nude mouse certainly suggests a tendency to promote a systemic *M. leprae* infection. If the problems of survival can be overcome, the nude mouse could prove to be of considerable value as a model for the study of lepromatous leprosy and as a means of detecting a small number of viable organisms against a large background of dead bacilli in the tissues of patients undergoing therapy.

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Control of lymphokine secretion by prostaglandins

SEVERAL lines of evidence exist implicating prostaglandins (PGs) as mediators in inflammation¹. In type IV hypersensitivity (delayed hypersensitivity, cellular immunity) reactions, although there is evidence of PG involvement^{2,3} lymphokines, the products of antigen-activated thymus-dependent lymphocytes, have received greater attention as potential mediators^{4,5}. It has been reported that PGE₁ is a potent inhibitor of lymphocyte activation *in vitro*⁶ and we have observed that guinea pig peritoneal exudate macrophages produce substantial amounts of E-type PG (ref. 7). It is well established that macrophages are capable of modifying lymphocyte activation^{8,9}, and that there is close association between these two cell types in reactions of cellular immunity *in vitro*¹⁰ and *in vivo*¹¹. We have now shown that production of E-type PGs by macrophages modifies the secretion of lymphokines by lymphocytes in response to antigen and that this may serve to provide a negative feedback mechanism for regulating the extent and duration of reactions of cellular immunity.

The *in vitro* test that shows the best quantitative correlation with delayed hypersensitivity reactions in the guinea pig is macrophage migration inhibition⁴ and so we have used this test both for measurement of the reactivity of sensitised lymphocytes to antigen and for the direct measurement of lymphokine activity. Antigen-induced cell migration inhibition of sensitised cells was reduced by PGE₁ (0.1-1.0 µg ml⁻¹), although the inhibition of cell migration by preformed lymphokine was not affected by PGs of the E series (Table 1), suggesting that lymphokine formation rather than lymphokine action on target cells was inhibited by PGE compounds. Neither PGF_{2α} nor PGA₁, at these concentrations, influenced antigen-induced cell migration inhibition (Table 1). On the other hand, indomethacin (0.1 µg ml⁻¹), a potent prostaglandin synthase inhibitor¹, enhanced antigen-induced inhibition, but had no effect on lymphokine-induced inhibition (Table 1), implying that there is sufficient endogenous PG biosynthesis in these cultures to modify the response to antigen. These observations support the proposition that endogenous E-type PGs act by reducing the lymphokine secretion by antigen-activated lymphocytes, rather than by modifying the action of lymphokines on target cells.

Accordingly, we have measured lymphokine production by lymph node lymphocytes in response to specific antigen, in the

Table 2 Effect of prostaglandins E₁ and E₂ and indomethacin on lymphokine formation measured by guinea pig cell migration inhibition

Inducing antigen	Drug	Response (% ± s.e.m.)		
		PGE ₁	PGE ₂	Indomethacin
-	-	100 ± 2.2	100 ± 2.2	100 ± 2.3
1,000	-	52.3 ± 1.5	57.8 ± 1.5	71.9 ± 2.3
1,000	0.1	64.7 ± 3.0	71.7 ± 1.6	65.9 ± 2.2
1,000	1.0	81.7 ± 4.9	81.6 ± 4.3	ND

Lymphocytes were obtained from the teased lymph nodes of BGG-sensitised animals. They were placed in culture tubes at 1×10^7 cells ml⁻¹ in Eagle's MEM supplemented with 5% pooled normal guinea pig serum. BGG (1 mg ml⁻¹) with or without E-type PGs were added to test cultures and after 24 h incubation at 37 °C, culture supernatants were partially purified and tested for migration inhibitory activity. Results were expressed as percentage response of control ± s.e.m. Supernatants without added antigen or drugs were taken as controls.

presence and absence of E-type PGs and indomethacin. PGE₁ (0.1-1.0 µg ml⁻¹) and PGE₂ (0.1-1.0 µg ml⁻¹) produced dose-related inhibition of antigen-induced lymphokine secretion, measured in terms of migration inhibitory activity (Table 2). These results are in agreement with the direct effects of PGE compounds on antigen-induced migration inhibition. We have obtained similar results to those shown in Tables 1 and 2 for mitogenic lymphokine, in that antigen-induced stimulation of DNA synthesis in lymph node lymphocytes was inhibited by PGE₁ and PGE₂ (0.1 and 1.0 µg ml⁻¹) and that this action of E-type PGs was accompanied by reduced production of mitogenic lymphokine.

Indomethacin (0.1 µg ml⁻¹) had only a slight enhancing effect on lymphokine secretion by lymph node lymphocytes (Table 2), whereas it clearly augmented antigen-induced inhibition of cell migration—a system which utilises mixed cell populations (that is, macrophages and polymorphonuclear (PMN) leukocytes, in addition to lymphocytes) (Table 1). These observations confirmed that the action of PGE was at the level of lymphokine secretion by lymphocytes, but implied that the source of endogenous E-type PGs was unlikely to be the lymphocytes themselves.

As a result of these findings, we have examined PG production by inflammatory cell populations (lymphocytes, macrophages and PMN leukocytes) during short term culture. Macrophage-rich cell populations (60-80% macrophages, 10-30% PMN leukocytes, 10% lymphocytes) produced substantial amounts of PGE-like activity (mean ± s.e. = 11.6 ± 2.1 ng PGE₂ per 10^6 cells per 24 h) whereas PMN leukocytes (80-90% leukocytes, 10-20% monocytes) and lymph node lymphocytes (>90% lymphocytes) were much less active in this respect (0.45 ± 0.03 ng and 1.7 ± 0.11 ng PGE₂ per 10^6

Table 1 Effects of prostaglandins and indomethacin on antigen- or lymphokine-induced guinea pig cell migration inhibition

Ag	Treatment (µg ml ⁻¹)		PGE ₁	PGE ₂	Response (% ± s.e.m.)		Indomethacin
	LK	Drug			PGF _{2α}	PGA ₁	
-	-	-	100 ± 3.0	100 ± 3.8	100 ± 6.0	100 ± 4.7	100 ± 4.4
-	-	0.1	99.9 ± 4.9	103.1 ± 4.4	102.6 ± 4.6	102.5 ± 2.6	96.0 ± 4.6
-	-	1.0	97.7 ± 1.9	94.5 ± 3.4	93.5 ± 4.9	95.8 ± 6.2	ND
5.0	-	-	67.3 ± 3.0	44.4 ± 0.4	41.8 ± 0.4	48.6 ± 3.8	69.4 ± 2.2
5.0	-	0.1	76.2 ± 3.9	55.1 ± 2.2	46.2 ± 4.3	46.7 ± 5.3	57.5 ± 3.9
5.0	-	1.0	88.9 ± 4.4	61.1 ± 2.5	43.3 ± 2.9	50.3 ± 4.6	ND
-	100	-	74.7 ± 2.9	71.5 ± 2.6	ND	ND	56.7 ± 1.6
-	100	0.1	73.6 ± 0.1	74.2 ± 4.9	ND	ND	58.1 ± 3.3
-	100	1.0	76.5 ± 4.1	73.1 ± 2.8	ND	ND	ND

Cell migration inhibition was carried out as described previously¹⁷. Guinea pig peritoneal exudate cells in Eagle's MEM with 10% foetal calf serum were cultured at 4×10^6 cells per capillary tube in chambers with or without antigen, lymphokine or drugs. Cell migration was recorded after 40 h as area (mm²) and then expressed as percentage inhibition of control (that is, with no addition) ± s.e.m. Purified protein derivative of tuberculin (PPD) was used as antigen (Ag) with tuberculin-sensitised animals (0.1 ml BCG intraperitoneally 4 weeks before use) and preformed lymphokine (LK) was generated from bovine gamma globulin (BGG) sensitive lymph node lymphocytes in culture with 1.0 mg ml⁻¹ BGG. Lymphokine preparations were partially purified by ammonium sulphate precipitation as described previously¹⁷.

Table 3 *In vitro* prostaglandin production by guinea pig inflammatory cell populations stimulated by lymphokine or specific antigen

Antigen ($\mu\text{g ml}^{-1}$)	PGE ₂ (ng per 10 ⁶ cells per 24 h)	Lymphokine ($\mu\text{g ml}^{-1}$)	PGE ₂ (ng per 10 ⁶ cells per 24 h)
0	7.60 $\pm$ 0.86*	0	6.87 $\pm$ 0.73*
0.01	6.22 $\pm$ 0.92	4.0	5.13 $\pm$ 0.16
0.1	9.24 $\pm$ 0.56	20.0	7.89 $\pm$ 0.48
1.0	11.58 $\pm$ 0.83	100.0	11.58 $\pm$ 1.43
10.0	20.38 $\pm$ 1.41	500.0	23.87 $\pm$ 1.89
100.0	26.00 $\pm$ 0.98		

*Mean $\pm$ s.e.m.

Sterile Falcon culture tubes containing 5×10^6 washed, macrophage-rich peritoneal exudate cells and increasing doses of specific antigen (PPD) or lymphokine were incubated at 37 °C for 24 h and the supernatants removed and stored at -20 °C before assay for PG activity by radioimmunoassay using sheep anti-PGE₂-BSA. Anti-PGE₂ antiserum cross reacted with PGE₂ (100%); PGE₁ (55%); PGF_{2 α} (1.5%); 15-keto PGE₂ (1.2%); PGA₂ (0.6%); PGB₂ (0.2%).

cells per 24 h, respectively). Macrophage populations (>90% macrophages) enriched by selection of glass-adherent cells show a similar PG-synthesising capacity to that of unseparated exudate cell populations, further implying that the macrophage is the major cell type responsible for PG production.

Prostaglandin-like activity was mainly E-type, as detected by radioimmunoassay, had the properties of an acidic lipid on extraction into ethyl acetate and cochromatographed with authentic PGE₂ on thin-layer chromatography. The biological activity of the material on rat stomach strip, human Fallopian tube and ADP-induced platelet aggregation suggested that the bulk of the activity resembled PGE₂ rather than PGE₁, a conclusion consistent with the high proportion of arachidonic acid (the PG₂ precursor) in macrophage membrane phospholipids¹². A dose-related increase in PGE production was induced by specific antigen using sensitised cells and by pre-formed lymphokine using non-sensitised cells (Table 3), thus satisfying an obligatory requirement of the negative feedback hypothesis. Furthermore, indomethacin at a concentration which completely inhibited PGE production (0.1 $\mu\text{g ml}^{-1}$) increased the formation of macrophage migration inhibitory lymphokine by antigen-stimulated peritoneal exudate cells from 19.7 $\pm$ 2.1% inhibition (untreated cells) to 29.53 $\pm$ 2.6% inhibition (treated cells), confirming that the level of PGE production is sufficient to modify lymphokine generation *in vitro*.

We have established here that E-type PGs inhibit lymphocyte activation and the secretion of lymphokines and that such preparations stimulate E-type PG production by macrophages. This is consistent with the proposition that E-type PG production in response to lymphokine stimulation provides a negative feedback mechanism for regulation of the cellular immune response. Inhibition of mediator secretion by PGs in allergic reactions is not restricted to type IV hypersensitivity reactions. Thus, PGE₁ and PGE₂ are both effective inhibitors of histamine secretion by human basophils in type I hypersensitivity¹³ and antigen-induced SRS-A production by lung fragments¹⁴. Consistent with this phenomenon is the observation that low doses of indomethacin enhance SRS-A release from passively sensitised human lung¹⁵. In these situations, in contrast to the type IV response, the cellular origin of PG production has not been clearly established. In type III allergic reactions, although PGE₁ can inhibit the release of lysosomal enzymes from PMN leukocytes¹⁶, the capacity of such cells to produce PGE seems to be insufficient for this effect to be of physiological relevance. The inability of therapeutic doses of non-steroidal anti-inflammatory drugs to affect markedly types I, III and IV hypersensitivity reactions *in vivo* implies that other control systems also operate in physiological, as opposed to pathological, circumstances.

The self-limiting mechanism that we propose for type IV reactions has the virtue of relating lymphokine, lysosomal enzyme and PG release to a common basis. This relationship may have interesting consequences, both for aetiology and pathogenesis in chronic inflammatory diseases¹⁶, and in the design of anti-

inflammatory drugs, by highlighting potential loci of action that have hitherto received little attention.

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Phorbol esters stimulate DNA synthesis and ornithine decarboxylase activity in mouse epidermal cell cultures

TUMOUR-PROMOTING agents have been defined by their ability to promote tumour formation on carcinogen-initiated mouse skin. The most potent of these agents are the diesters of phorbol which are the active components of croton oil, the classic promoting substance. Phorbol esters can be chemically modified in a number of positions to form molecules with a wide range of promoter potency¹. Such modifications have been useful in determining structure-function relationships². A good correlation exists between the ability of phorbol esters to promote epidermal tumours and their ability to stimulate epidermal macromolecular synthesis *in vivo*^{3,4}. In particular, all tumour promoters stimulate synthesis of epidermal DNA, although not all chemicals which induce hyperplasia are promoters¹. Recently O'Brien *et al.*⁵ suggested that the degree of induction of ornithine decarboxylase (ODC) activity in mouse skin after application of promoting and non-promoting compounds correlates well with their promoting potency. Evidence from our laboratory indicates that brief exposure to the potent phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA), stimulates DNA synthesis and

ODC activity in mouse epidermal cell cultures (S.H.Y., T.B., E.P., D. Michael, K. Elgjo and H.H., to be published, and U.L. and S.H.Y., to be published). Using such a culture system, we have examined a series of phorbol esters to test the correlation of promoter potency *in vivo* with the stimulation of DNA synthesis and ODC activity *in vitro*.

Mouse epidermal cells were isolated and cultured as previously reported^{6,7}. Phorbol esters were dissolved in DMSO as stock solutions of 1 mg ml⁻¹ or 0.1 mg ml⁻¹ and stored at -70 °C. Experimental media were prepared immediately before use. In most experiments cells were exposed to the promoter for 1 h (except for phorbol which was kept continuously in medium), starting 20–24 h after plating. After removal of the promoter, cells were washed with phosphate-buffered saline and reincubated in complete medium (CM)⁷, which was changed daily for the assay of DNA synthesis.

The dose-response curve for stimulation of DNA synthesis in cells treated with the relatively strong promoter 12-*O*-hexadecanoylphorbol-13-acetate (HPA) is shown in Fig. 1a. At all doses tested there was an initial inhibition of DNA synthesis at 24 h after the 1-h pulse treatment, followed by a return to near-normal or elevated levels by 2 d. The highest dose (1 µg ml⁻¹) tested gave the largest initial inhibition. By day 3 after treatment, effective doses produced a dose-dependent stimulation with a maximum usually occurring on day 4. Cells responded to doses as low as 0.05 µg ml⁻¹ (8 × 10⁻⁹ M). Exposure to more than 0.5 µg ml⁻¹ seemed to reduce the ultimate stimulation, perhaps because of increased initial toxicity. These results, however, were col-

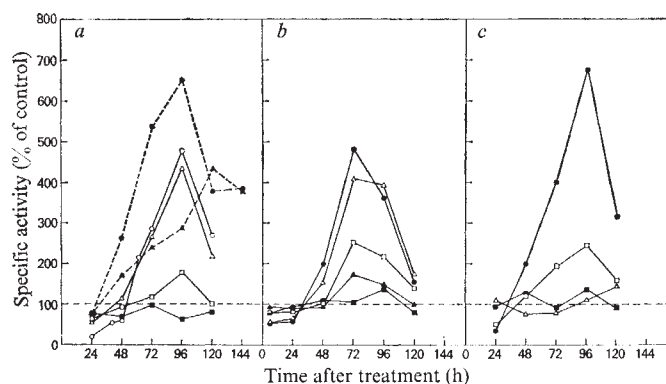


Fig. 1 Promoter-stimulated DNA synthesis. Epidermal cells were seeded at 7.5×10^6 cells per dish into 100-mm culture dishes. Twenty-four hours after plating, cultures were exposed to medium containing phorbol esters or vehicle (DMSO 0.1%) for 1 h, washed, reset with growth medium and changed daily. Concentration of the phorbol esters are given in µg per ml of culture medium. In the case of phorbol treatment (c), the experimental medium remained continuously, with a fresh solution prepared daily. At indicated intervals after treatment, dishes were pulsed with 1 µCi ml⁻¹ methyl-³H-thymidine (6 Ci mmol⁻¹, Schwarz-Mann, Orangeburg, New York) for 1 h. Labelled cells were washed three times with cold phosphate-buffered saline containing 0.1% unlabelled thymidine, scraped from the dishes, centrifuged and the pellets frozen. Pellets were washed three times with 0.2 N PCA and hydrolysed in 0.5 N PCA at 90 °C for 20 min. The DNA content of each hydrolysate was determined by the diphenylamine reaction¹⁰ and radioactivity by counting an aliquot in Hydromix (Yorktown Research, New Hyde Park, New York) in a Beckman LS 250 scintillation counter. Results are expressed as the specific activity (c.p.m. per µg DNA) of the treated groups relative to the DMSO control. All data represent the average of at least duplicate dishes. The variation in control values for each point within experiments was less than 20%. a, Data collected on different primary cell isolates represented by ---, HPA 0.5 (●) and 0.05 (▲) µg ml⁻¹; —, HPA 1.0 (○), 0.1 (△), 0.01 (□) and 0.001 (■) µg ml⁻¹. The time course and maximum stimulation of DNA synthesis varies somewhat for different primary cell populations. b: ●, TPA 0.1 µg ml⁻¹; △, PDBe 1.0; ▲, PDBe 0.1; □, PDBu 1.0; ■, PDBu 0.1. c: ●, TPA 0.1 µg ml⁻¹; □, MeTPA 1.0; ■, MeTPA 0.1; △, phorbol 1.0.

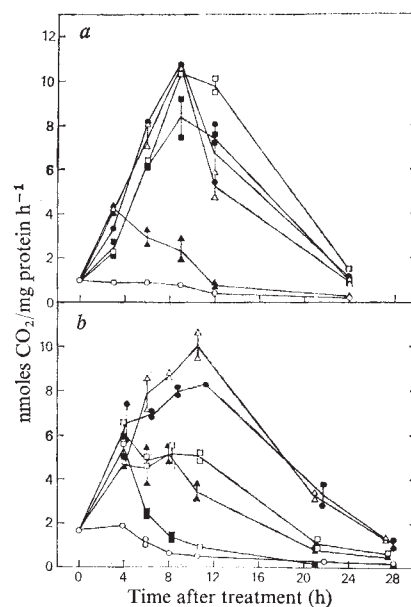


Fig. 2 Epidermal ornithine decarboxylase activity after 1-h treatment with phorbol esters. Cells were plated at 3×10^6 cells per 60-mm culture dish and treated at 21 h (a) and 23 h (b). a: ●, TPA 0.1 µg ml⁻¹; □, HPA 1.0; ■, HPA 0.1; △, PDBu 1.0; ▲, PDBu 0.1; ○, DMSO. b: ●, TPA 0.1 µg ml⁻¹; □, MeTPA 1.0; ■, MeTPA 0.1; △, PDBe 1.0; ▲, PDBe 0.1; ○, phorbol 1.0. At indicated times after the start of the treatment, duplicate dishes were washed with PBS and stored frozen until assayed. Cells were scraped from dishes and suspended in 300 µl PBS. ODC was measured on 50-µl aliquots of the cell lysate in a final volume of 100 µl containing 0.25 µmol dithiothreitol, 0.1 µmol EDTA, 0.01 µmol pyridoxal phosphate, 5.0 µmol sodium phosphate, pH 7.2 and 0.1 µCi L-1-¹⁴C-ornithine (2.3 mCi mmol⁻¹, diluted from DL-1-¹⁴C-ornithine HCl, New England Nuclear, Boston, Massachusetts, 54 mCi mmol⁻¹, with non-radioactive L-ornithine). The incubation was carried out for 1 h at 37 °C in 17 × 100-mm Falcon culture tubes capped with serum stoppers equipped with polypropylene centre wells (Kontes Glass Co., Vineland, New Jersey) containing a filter paper wick. The reaction was stopped with 0.2 ml 2 N perchloric acid and ¹⁴CO₂ was trapped by 0.2 ml NCS (Amersham/Searle Corp., Arlington Heights, Illinois) in the centre well during an additional 1-h incubation. The centre well and its content was counted in 10 ml Spectrafluor (Amersham/Searle) in toluene. Counts were corrected for blanks that contained all components except the cell lysate. Protein was measured on 50-µl aliquots of the cell lysate by the method of Lowry as detailed by Layne¹¹. Each point on the graph represents the average of duplicate determinations of specific enzyme activity on cells from a single culture dish (variation < 10%). In b the DMSO control and phorbol-treated culture values are superimposed.

lected from experiments carried out on separate cell isolates (solid lines and dashed lines), making absolute dose-response comparisons across experiments difficult to interpret accurately. Similar studies using TPA as promoter¹⁴ demonstrated a dose-dependent stimulation of DNA synthesis at doses between 10⁻⁸ and 10⁻⁹ M.

When the less potent tumour-promoting phorbol esters, phorbol dibenzoate (PDBe) and phorbol dibutyrate (PDBu), are compared with TPA for ability to stimulate DNA synthesis (Fig. 1b), one finds that at approximately equimolar doses, PDBe and PDBu are much less effective than TPA. At 0.1 µg ml⁻¹ PDBu induces no stimulation, whereas PDBe stimulates only slightly. At doses 10 times higher than TPA, the stimulation with PDBe is equivalent to TPA, whereas that with PDBu is less than half of that found with TPA. Phorbol itself, a non-promoter for mouse skin, failed to stimulate even at a dose of 1 µg ml⁻¹ (Fig. 1c). But 4-*O*-tetradecanoylphorbol-13-acetate (MeTPA), also reported to be a non-promoter for skin carcinogenesis⁸, induced an initial inhibition followed by a twofold stimulation of DNA

Table 1 Correlation between potency of phorbol esters for promoting skin tumours and their ability to stimulate DNA synthesis and induce ODC activity in epidermal cells *in vitro*

Phorbol ester	Molecular weight	Tumour-promoting potency (1/TD ₅₀)*	Maximum stimulation of DNA synthesis <i>in vitro</i> †		Maximum induction of ODC <i>in vitro</i> †	
			0.1 µg ml ⁻¹	1 µg ml ⁻¹	0.1 µg ml ⁻¹	1.0 µg ml ⁻¹
TPA	616.8	15.4	1.00	—	1.00	—
HPA‡	644.8	5.4	—‡	—	0.76	0.97
PDBe	572.7	<1.5§	0.19	0.82	0.55	1.3
PDBu	504.6	0.1	0.11	0.40	0.34	0.95
MeTPA	630.8	Non-promoter	0.07	0.26	0.44	0.51
PDA¶	448.5	Non-promoter	0	0	0	0
Phorbol	364.4	Non-promoter	—	0	0	0

*TD₅₀ = total dose (µmol) of promoter required to produce papillomas on 50% of CD-1 mice previously initiated with 51 µg 7,12-dimethylbenz(a)anthracene. Data for TPA, HPA, PDBe, PDA and phorbol derived from ref. 12; data for PDBu derived from ref. 13; data for MeTPA from ref. 8 and mouse strain was not identified.

†Maximum stimulation did not always occur at same time after treatment.

‡DNA synthesis after HPA and TPA was not studied simultaneously in the same cell preparations. Since variations in absolute responses occur in different cell preparations (see Fig. 1b and c), direct comparisons between HPA and TPA cannot confidently be made. Based on experience with a number of separate experiments, TPA induces 20–30% more stimulation than HPA at approximately equimolar doses.

§Data available only for 20% tumour incidence in CD-1 mice (see ref. 9).

¶Phorbol-13,20-diacetate calculated from data reported in ref. 14 and unpublished work of S.H.Y., T.B., E.P., D. Michael, K. Elgjo and H.H.

Tumour-promoting potency was assigned as indicated from appropriate literature citation. Capacity to stimulate DNA synthesis and induce ODC activity was measured using data in Figs 1 and 2, or as otherwise noted. Comparisons were made only with data from experiments performed on the same primary cell isolate. For both indices the maximum level of stimulation induced by TPA after subtraction of vehicle control levels, was assigned a value of 1.00. Other compounds are presented as the ratio of their net values (maximum stimulation minus vehicle control) to that of TPA. Repeated experiments indicate that the absolute ratios presented would vary from experiment to experiment, but the relative rankings would be constant.

synthesis at a dose of 1 µg ml⁻¹ (Fig. 1c). At 0.1 µg ml⁻¹ it was ineffective. This compound has also been reported to inhibit replicative and repair DNA synthesis in human fibroblast cultures at doses greater than 1 µg ml⁻¹ (ref. 8).

Induction of ornithine decarboxylase activity has been proposed as a marker for promoter activity *in vivo*⁵ and *in vitro* (our unpublished work). The results in Fig. 2 demonstrate the relative effectiveness with which a series of phorbol esters induce ODC in epidermal cells *in vitro*. At a dose of 0.1 µg ml⁻¹, TPA induces a 5–10-fold rise in activity within 6–12 h of exposure, with a return to near-normal values by 24–28 h. The time course and maximum level of stimulated enzyme activity varies somewhat for different primary cell populations. As seen for stimulation of DNA synthesis, HPA (1 µg ml⁻¹ and 0.1 µg ml⁻¹), PDBu (1 µg ml⁻¹) and PDBe (1 µg ml⁻¹) induced a similar rise in ODC activity, whereas PDBu (0.1 µg ml⁻¹), PDBe (0.1 µg ml⁻¹) and MeTPA (1 µg ml⁻¹) were less effective as inducers. Phorbol (1 µg ml⁻¹), phorbol-13,20-diacetate (1 µg ml⁻¹, not shown) and the DMSO vehicle were completely ineffective in inducing ODC. The lower dose of MeTPA (0.1 µg ml⁻¹) caused a transient early rise in activity which returned to control values by 8 h. The accelerated kinetics of induction and decay seen with 0.1 µg ml⁻¹ MeTPA and PDBu were at doses which failed to stimulate DNA synthesis; the significance of this observation remains to be determined.

These results indicate that there is a good correlation between the potency of phorbol esters for promoting skin tumours *in vivo* and their ability to stimulate DNA synthesis and induce ODC activity in epidermal cells *in vitro* (Table 1). The molecular weights of all compounds other than HPA and MeTPA are lower than that of TPA. Therefore the molar concentrations at which they were tested exceeded the TPA concentration. Only HPA seems to approach the effectiveness of TPA in stimulating both DNA synthesis and ODC activity at the same molar concentration. For MeTPA, results *in vitro* suggest that it has biological activity and should be retested as a promoter *in vivo*, preferably on mice sensitive to formation of skin tumours⁹, before it is accepted as a negative control for TPA. Although promoter and non-promoter compounds outside the phorbol family must be tested before more general conclusions are reached, mouse epidermal cell cultures seem to be a useful system for identifying and ap-

praising potential promoting agents. Additionally, the similarity between *in vivo* and *in vitro* results indicates that this model should be useful for investigating the cellular mechanisms of promoter action and for studying two-stage carcinogenesis *in vitro*.

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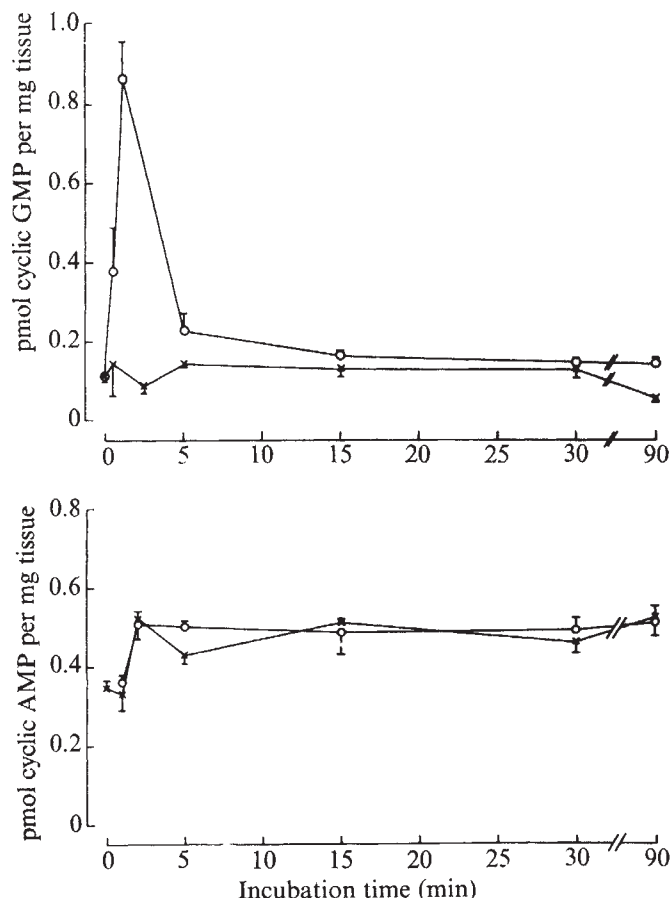
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Intracellular messenger role of cyclic GMP in exocrine pancreas

THE response of cells to hormones and neurotransmitters is mediated by chemical regulators, of which calcium, adenosine cyclic 3',5'-monophosphate (cyclic AMP) and guanosine cyclic 3',5'-monophosphate (cyclic GMP) are considered to be of primary importance. The endogenous occurrence of



cyclic GMP was discovered more than 10 yr ago, but until recently its formation has been reported chiefly in association with the activation of cholinergic muscarinic receptors^{1,2}. The description in the pancreas of a cyclic GMP-dependent protein kinase³, a cyclic GMP phosphodiesterase⁴, and the ability *in vivo* of cholecystikinin to

Fig. 2 Stimulation of enzyme secretion from isolated guinea pig pancreatic slices by CCK. Tissue was incubated as described for Fig. 1. The esterase activity of the kallikrein-trypsin-like proteases released into the medium was determined by hydrolysis of the synthetic substrate, benzoyl-L-arginine-ethyl ester (BAEE), after activation of the proenzymes by enterokinase. A sample 0.5 ml of enzyme solution was incubated with 0.1 ml enterokinase (50 μ g) for 10 min at 37 °C before assay. The accumulated proenzyme activity is expressed as BAEE ΔE_{366} U per mg wet weight tissue, with 1 U equivalent to an absorbance change recorded as absorbance units per min per ml of enzyme solution $\times 10^6$. Vertical bars indicate 1 s.e.m. $\times$, Control; $\circ$, CCK (GIH, 1 U ml⁻¹); Δ , secretin (GIH, 1 U ml⁻¹).

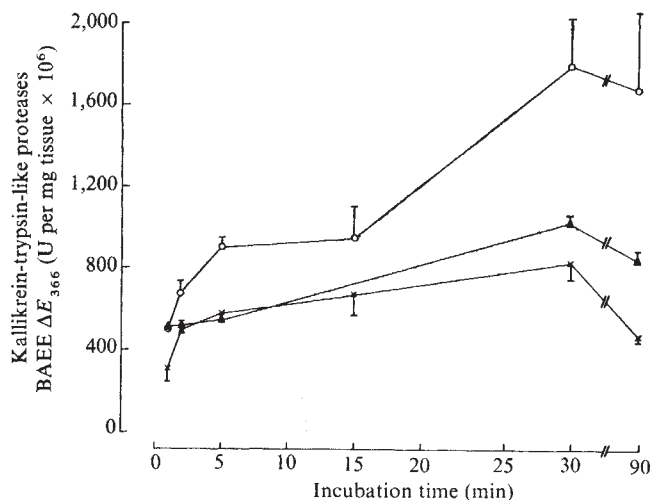


Fig. 1 Time course of cyclic GMP and cyclic AMP levels in isolated guinea pig pancreatic slices produced by CCK (1 U ml⁻¹; GIH preparation). Pooled pancreatic slices were initially incubated for 15 min. Three or four slices of total weight between 80–110 mg were then incubated in individual Erhlemeyer flasks containing 2 ml of Krebs–Ringer bicarbonate buffer with 0.5% bovine serum albumin. Each flask was gassed with 95% O₂–5% CO₂. After preincubation for 15 min, CCK was added to the flask. On completion of incubation the slices were removed and frozen in an aluminium clamp precooled in liquid nitrogen. The frozen tissue was dropped into 0.5 ml of 6 mM theophylline at 100 °C and boiled for 10 min. The denatured tissue slices were homogenised fully. The homogenate was stored overnight at –20 °C, after addition of 0.5 ml of acid ethanol (1% HCl in absolute ethanol). The samples were centrifuged at 1,500g for 5 min and the supernatants were decanted. Each precipitate was washed with 0.5 ml of acid alcohol and, after respinning, the corresponding supernatants were pooled. The extracts were taken to dryness at 50 °C and the dried residue was resuspended in 0.5 ml of 50 mM Tris–HCl buffer, pH 7.4. Samples of 50 μ l were used for measuring cyclic GMP and cyclic AMP. Cyclic AMP was measured by a binding protein method^{6,7}. Cyclic GMP values were determined using a rabbit antiserum (HO/2/75) raised to succinylated cyclic GMP conjugated to human serum albumin⁸. A sample of 100 μ l of antiserum diluted 1:1,000 in Tris buffer (50 mM, pH 7.4), 50 μ l of tritiated cyclic GMP, specific activity 21 Ci mmol⁻¹, containing 3 nCi of ³H-cyclic GMP, and 50 μ l of cyclic GMP standards (0–3 pmol), or unknown samples, were incubated at 4 °C for 12 h. Free and antibody-bound ³H-cyclic GMP were separated by filtration through a 0.45- μ m Millipore filter. Filters were then extracted in a toluene–methoxyethanol-based scintillant, and the antibody-bound ³H-cyclic GMP was measured in a liquid scintillation spectrometer. Cyclic AMP in a concentration of 1 pmol ml⁻¹ showed a cross reaction in the assay of less than 0.2%, and other cyclic nucleotides less than 0.01%. Nucleotides, and nucleotide monophosphates, diphosphates and triphosphates, showed virtually no cross reaction up to a concentration of 10⁴ pmol ml⁻¹. Dilutions of pancreatic cyclic GMP showed parallelism with dilutions of standard cyclic GMP. As a further check that the immunoreactive material was identical with cyclic GMP, control pancreatic slices and slices stimulated with acetylcholine were extracted using the standard trichloroacetic acid procedure. The extracts were then divided and one portion of each was treated with 50 μ l of beef heart cyclic nucleotide phosphodiesterase (Sigma), at a concentration of 1 mg ml⁻¹, for 60 min at 37 °C and then boiled for 4 min. Standard cyclic GMP was treated similarly. The immunoreactive cyclic GMP in the control and stimulated samples were destroyed to the same extent as the standard cyclic GMP. Each experimental point represents the mean of three incubations. The tissue cyclic nucleotide content of each incubation sample was measured in triplicate. The cyclic nucleotide values are calculated as pmol of cyclic nucleotide per mg wet weight pancreatic tissue. The vertical bars indicate 1 s.e.m. $\times$, Cyclic GMP levels; $\circ$, cyclic AMP levels; $\times$, control; $\circ$, CCK (1 U ml⁻¹).

increase cyclic GMP levels⁵, suggested a physiological role for cyclic GMP in the mediation of hormone action. We report here experiments on isolated guinea pig pancreatic slices, in which the hormone cholecystikinin (CCK) and the neurotransmitter acetylcholine (ACh) both produced a marked, transient increase in the tissue level of cyclic GMP, which preceded the release of secretory enzymes. Exposure of the isolated pancreatic slices to the hormone secretin evoked a more prolonged increase in cyclic AMP, without any significant effect on cyclic GMP.

CCK (GIH Research Unit, Karolinska Institute), highly purified CCK (given by Professor Viktor Mutt), and the synthetic C-terminal octapeptide of CCK (given by Dr Miguel Ondetti, Squibb Institute), all produced within seconds a rapid increase in cyclic GMP that reached peak values at 2 min and fell rapidly to near the control level by 5 min (Fig. 1). The increase in cyclic GMP preceded any significant acceleration in the release of zymogens (Fig. 2). ACh evoked a pattern of increase in cyclic GMP that was similar to that obtained with CCK with regard to time course and relationship to onset of enzyme release. The increase in cyclic GMP triggered by ACh (10⁻⁷–10⁻³ M) and CCK (5 $\times$ 10⁻⁹–1 $\times$ 10⁻⁷ M) was dose related. Atropine abolished the ACh-mediated zymogen release and increase

in the tissue level of cyclic GMP (Fig. 3), but did not affect the CCK response. Both ACh and CCK failed to alter significantly the basal cyclic AMP values in actively secreting pancreatic slices. In the presence of the phosphodiesterase inhibitors, theophylline (3 mM) and 3-isobutyl-1-methylxanthine (IBMX, 0.5 mM), basal levels of cyclic AMP were increased about fivefold, but no further increase was observed with CCK. In contrast, the basal levels of cyclic GMP were increased to a lesser extent (about two-fold) by theophylline and IBMX and the increase in cyclic GMP caused by CCK was potentiated. In three separate experiments, the synthetic analogue 8-bromo cyclic GMP (1 mM) increased enzyme secretion over control values by 27.5% ($P < 0.025$) in pancreatic slices incubated for 30 min.

Secretin, in the presence of trasyolol (260 U ml^{-1}), produced a rapid increase in cyclic AMP that reached a maximum at 1 min but remained elevated for at least 30 min and returned to basal values at 90 min (Fig. 4). Secretin did not significantly alter cyclic GMP levels, even in the presence of theophylline (3 mM) and trasyolol (260 U ml^{-1}).

Enzyme secretion from pancreatic exocrine acinar cells is stimulated by CCK and the vagus nerve (ACh). Activation by CCK and ACh of specific receptors on the plasma membrane of acinar cells results in a rapid, marked, but transient increase in cyclic GMP. This may trigger the intracellular mobilisation of ionised calcium, which is considered to initiate the release of zymogens from acinar cells. Alternatively, it is possible that the increase in cyclic GMP results from, rather than precedes, the changes in intracellular calcium. The difference in the time courses of cyclic GMP and the accumulated enzyme secretion may be explained by the fact that the transient increase in cyclic GMP, through activation of a cyclic GMP-dependent protein kinase³, may trigger intracellular release of ionised calcium which then stimulates the enzyme secretion; this response once initiated may be maintained by

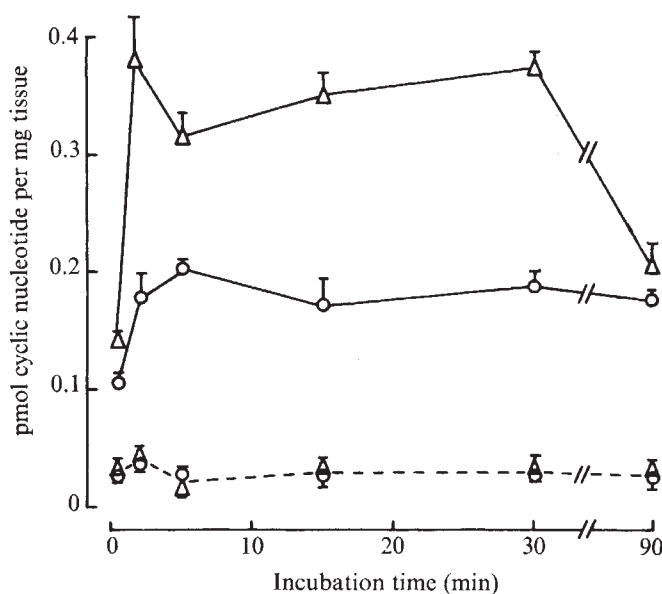


Fig. 4 Time course of cyclic AMP and cyclic GMP formation in guinea pig pancreatic slices after addition of secretin. Tissue incubation and cyclic nucleotide measurement were as described in Fig. 1, except that the Krebs-Ringer buffer solution contained trasyolol (260 U ml^{-1}). Levels of cyclic nucleotides are expressed as pmol per mg wet weight. The vertical bars represent 1 s.e.m. ○, Control; △, secretion (1 U ml^{-1}); —, cyclic AMP; ---, cyclic GMP.

regenerative release of calcium or by some other molecular mechanism. The secretion of electrolytes and water that accompanies enzyme release is primarily controlled by the hormone secretin. The interaction of secretin with cell receptors activates adenylate cyclase¹⁰, resulting in increased formation of cyclic AMP. The sequential molecular events through which cyclic nucleotides stimulate enzyme and electrolyte secretion are still obscure. Our results strongly suggest that in the exocrine pancreas each of the two major physiological functions is modulated by a specific cyclic nucleotide, enzyme secretion by cyclic GMP, and fluid and ion secretion by cyclic AMP. Dysfunction of the molecular mechanisms involved in the turnover of the two messengers may be of considerable importance in the aetiology of pancreatic disease.

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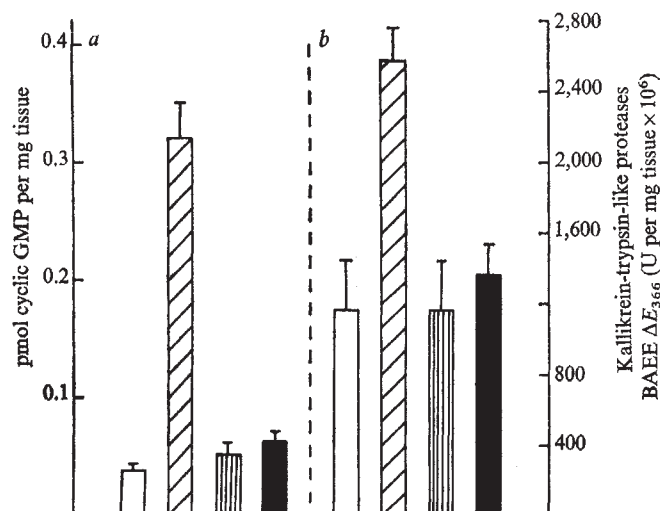
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Fig. 3 Histogram of the effect of atropine on the increase in cyclic GMP and enzyme secretion evoked by acetylcholine. Isolated pancreatic slices were incubated in Ehrlenmeyer flasks as described in Fig. 1. Atropine (10^{-6} M) was added to the appropriate Krebs-Ringer buffer solution at the beginning of the 15 min of preincubation. Open columns, control; diagonally hatched columns, acetylcholine (10^{-6} M); vertically hatched columns, atropine (10^{-6} M); closed columns, atropine (10^{-6} M) + acetylcholine (10^{-6} M). The vertical bars represent 1 s.e.m. *a*, Cyclic GMP content of pancreatic slices after 1 min of incubation with drug, expressed as pmol per mg wet weight tissue. *b*, Accumulated release of kallikrein-trypsin zymogens after 30 min into the incubation medium, expressed as BAEE ΔE_{366} units per mg wet weight of tissue, 1 U being equivalent to an absorbance change recorded as absorbance units per min per ml of enzyme solution $\times 10^6$.



Role of oestrogen receptor in androgen-induced sexual differentiation of the brain

THE sexual differentiation of gonadotropin secretion¹ and female sexual behaviour² occurs in the rat hypothalamus³ during the early postnatal period and is produced by testicular androgen in the male, although it may be mimicked in the female by administration of oestrogen or androgen³. The mechanism of this neural differentiation probably involves the aromatisation of testicular androgen to oestrogen in the hypothalamus⁴, followed by a complex series of biochemical events contingent on the binding of this oestrogen to a receptor and the translocation of the steroid-receptor complex to the nucleus. Cytosol oestrogen receptors⁶⁻⁸ and aromatising enzymes⁴ have been demonstrated in the neonatal brain; aromatising enzymes are localised to the differentiating areas⁴ whereas oestrogen receptors are found throughout the forebrain^{6,7}. In the study reported here, we have measured the nuclear oestrogen receptor in the neonatal brain using a nuclear exchange assay⁹ and have found that its distribution is consistent with the postulated role of the oestrogen receptor in the sexual differentiation of the brain.

Two regions of the 4-d-old brain were used, one containing the ventral half (HYP) and the other the dorsal half (CORT) of all brain between two coronal cuts, one immediately rostral to the optic chiasma and the other immediately caudal to the mammillary bodies.

Figure 1 shows the exchange of ³H-oestradiol in purified nuclei prepared from the HYP region of neonatal males. Saturable, diethylstilboestrol (DES)-suppressible, exchanged ³H-oestradiol was found in nuclei from the neonatal male HYP region with a K_d of 3.4×10^{-10} M, a value typical of oestrogen receptors¹³ and close to the K_d for oestradiol of the cytosol oestrogen receptor from neonatal rat brain⁶.

Table 1 shows that 2.4×10^9 sites per mg of DNA were found in the neonatal HYP region, but none in the male CORT region or either region of the female brain. To test the hypothesis that the steroid responsible for the nuclear translocation of the oestrogen receptor in the male hypothalamus was secreted by the testis, nuclear sites were assayed in male rats 24 h after castration on day 3. No sites were detectable in either brain region of these animals. We then measured nuclear oestrogen receptor in females injected systemically with 50 μ g of testosterone. This gave an identical distribution of nuclear receptor to that seen in the neonatal male but with about three times the number of sites found in the male HYP region (Table 1).

Thus nuclear oestrogen receptor sites appear solely in the HYP region under the influence of the testis or exogenous testosterone and this is consistent with their organising effect on the neonatal hypothalamus being exerted through the oestrogen receptor. These findings substantiate our previous study

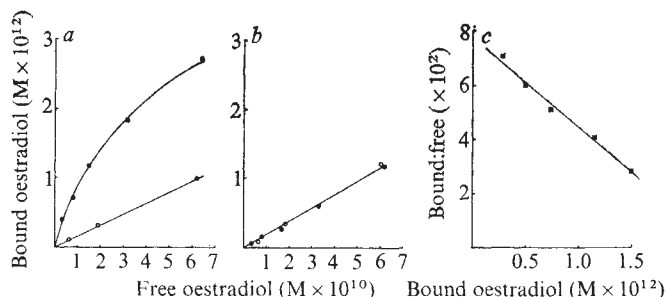


Fig. 1 The exchange of ³H-oestradiol in nuclei from the HYP region (a) and CORT region (b) of 4-d-old male rats. Purified nuclei were prepared by the method of Plapinger and McEwen¹² and samples (about 200 μ g of DNA), suspended in 0.4 ml buffer (1 mM phosphate, 0.32 M sucrose, 3 mM MgCl₂, pH 6.5) were incubated with various concentrations of ³H-oestradiol (95 Ci mmol⁻¹) for 1 h at 37 °C as described by Anderson *et al.*⁹. A separate series of tubes was incubated in the presence of a 200-fold molar excess of DES for the determination of non-specifically bound ³H-oestradiol. After incubation, the nuclei were washed four times by sedimentation (800g for 10 min) and resuspension for 25 min at 0–2 °C in 1 ml of fresh buffer. Radioactivity in the washed nuclei was measured, with an efficiency of 50%, after overnight extraction into 10 ml of scintillant (PPO 4 g l⁻¹; POPOP, 0.1 g l⁻¹ in toluene). Specific binding was determined by subtraction of nonspecifically bound ³H-oestradiol (○) from total bound ³H-oestradiol (●) and plotted according to Scatchard¹⁰ (c, male HYP region only), to give the dissociation constant (K_d) and number of specific binding sites (N_s). Samples of nuclei were analysed for DNA by the method of Burton¹¹.

in which nuclear binding was measured after injection of radioactive steroid¹⁴. The low affinity of testosterone for the neonatal brain oestrogen receptor⁶ and various studies on aromatisation in the neonatal brain^{4,15} suggest that obligatory aromatisation precedes the binding of testosterone to the oestrogen receptor.

After intracerebral injection of 0.27 μ g of DES, nuclear oestrogen receptor was found in similar amounts in the HYP and CORT regions of both male and female brain (Table 1). The number of sites was four to five times that in the male HYP region and we find this is the maximum number of sites obtainable by intracerebral injection of DES. The distribution of nuclear oestrogen receptor after intracerebral injection of DES is identical to that of the cytosol receptor in the untreated animal, that is, it is found throughout the forebrain^{6,7}. This is in marked contrast to the distribution of nuclear receptor after testosterone injection which mirrors the localisation of the aromatase enzymes, that is, nuclear receptor is found only in the HYP region. Thus there seems to be no inherent difference between the HYP and CORT receptors except in their proximity to aromatising enzymes. Cortical receptors are translocated to the nucleus when exposed to oestrogen and so may be important in some undefined aspect of brain development occurring when oestrogen levels are high, for example, prenatally or around day 10 of life in the female¹⁶.

It is probable, from the lack of nuclear oestrogen receptor in the CORT region of the untreated male and both regions of

Table 1 Distribution and dissociation constant of nuclear-bound oestrogen receptor in purified nuclei from brain of untreated and steroid-injected 4-d-old rats

	HYP		CORT	
	N_s per mg DNA ($\times 10^{-9}$)	$K_d \times 10^{10}$ (M)	N_s per mg DNA ($\times 10^{-9}$)	$K_d \times 10^{10}$ (M)
4-d-old male	2.40 ± 0.15	3.41 ± 0.30	*	*
4-d-old male, castrated day 3	*	*	*	*
4-d-old female	*	*	*	*
4-d-old female 3 h after 50 μ g testosterone subcutaneously in oil (50 μ l)	7.20 ± 0.46	2.01 ± 0.37	*	*
4-d-old female 1 h after 0.27 μ g DES intracerebrally in saline (10 μ l)	8.78 ± 0.90	3.52 ± 0.37	8.95 ± 0.38	4.27 ± 0.95
4-d-old male 1 h after 0.27 μ g DES intracerebrally in saline (10 μ l)	12.05 ± 2.66	4.66 ± 0.53	11.94 ± 2.25	4.57 ± 0.53

Number of sites (N_s) and dissociation constant (K_d) were obtained as described in Fig. 1.

*Undetectable.

the untreated female and castrate male, that no receptor has been translocated by circulating oestrogen. This is consistent with there being very little oestrogen circulating in the 4-d animal¹⁶, and any oestrogen present being sequestered by low affinity binding proteins of the blood and brain¹⁷. We believe that measurement of the nuclear receptor is likely to show where a steroid is acting, in contrast to the measurement of the cytosol receptor which merely shows where a steroid has the potential to act. In addition, we show that target tissue metabolism of a steroid may restrict nuclear translocation to a subset within a population of receptors, thereby restricting the action of the steroid to a few cells of a target organ though many may contain the receptor; this may be of relevance to other steroid sensitive tissues.

In conclusion, the distribution of the nuclear oestrogen receptor contrasts markedly with that of the cytosol receptor in the neonatal brain and is entirely consistent with the postulated roles of the oestrogen receptor and aromatisation in the androgen-induced sexual differentiation of the brain.

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2-Amino-4-phosphonobutyric acid as a glutamate antagonist on locust muscle

The characterisation of glutamate receptors in nerve and muscle membranes requires compounds which interact specifically with these receptors. In the vertebrate central nervous system various substances are reported to act as agonists or antagonists of glutamate (reviewed in refs 1 and 2). At the locust and crustacean nerve-muscle junctions, where the receptors are readily accessible for biochemical and pharmacological characterisation, comparatively few compounds activate the glutamate-sensitive receptors or antagonise the excitatory action of glutamate³⁻⁹ and so such substances are needed. We report here that the glutamate analogue, DL-2-amino-4-phosphonobutyric acid (DL-APB) (Fig. 1), inhibits the binding of glutamate to 'receptor-like' hydrophobic proteolipids isolated from locust muscle^{10,11} and antagonises the excitatory action of glutamate applied iontophoretically to receptors present in the locust muscle membrane. The shape and charge distribution of DL-APB are appropriate for interaction with glutamate receptors^{2,7,12}.

Proteolipids were extracted from approximately 2 g of the total intrathoracic muscle of locusts (*Schistocerca gregaria*)¹⁰. U-¹⁴C-L-glutamate (Amersham) was added to the proteolipid preparations which were then incubated at room temperature for 20 min before chromatography on a column (20×2 cm) of Sephadex LH 20 equilibrated with chloroform¹⁰. The effect of DL-APB (Calbiochem) on the binding of glutamate was measured by preincubating the

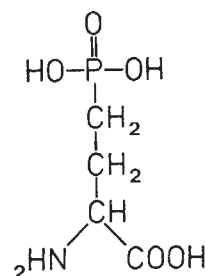


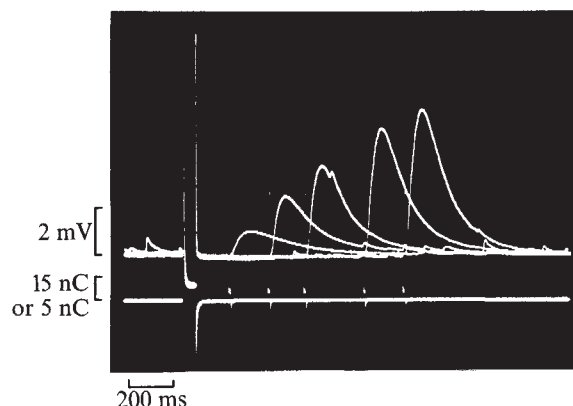
Fig. 1 2-Amino-4-phosphonobutyric acid.

proteolipid with DL-APB for 1 h before addition of U-¹⁴C-glutamate¹¹. Electrophysiological experiments were made on the extensor tibiae muscle, where junctional glutamate receptors are accessible to drugs applied iontophoretically¹³. Double and multi-barrel micropipettes of high resistance (200-300 MΩ)^{13,14}, with braking currents of 4×10^8 - 6×10^9 A were used to apply L-glutamate (Na salt, 0.1 M, pH 8) and DL-APB (Na salt, 0.1 M, pH 7) to glutamate receptors in the muscle membrane. In some experiments drugs were applied from two separate micropipettes, to exclude the possibility that interaction between barrels of the micropipette was affecting the observations.

Experiments on isolated proteolipids were made over a range of glutamate concentrations from 3.7×10^{-6} M to 1.0×10^{-4} M. Glutamate binding to the proteolipid was obtained by measuring the amount of glutamate associated with the peak of proteolipid eluted with chloroform^{10,11}. Saturation of binding occurred between 6.6×10^{-5} and 1.0×10^{-4} M when 7.25 nmol glutamate per mg proteolipid were bound. The dissociation constant, K_d , of the binding site was 8×10^{-6} M. The effect of DL-APB on the binding of glutamate to the proteolipid was measured over a range of glutamate concentrations (3.7×10^{-6} - 3.8×10^{-5} M) and over a range of DL-APB concentrations (1.0×10^{-5} - 5.1×10^{-3} M). Lineweaver-Burke and Dixon plots revealed that DL-APB behaves as a competitive inhibitor of glutamate binding with an apparent dissociation constant, K_d , of 6.6×10^{-5} M.

The iontophoretic application of glutamate to junctional glutamate receptors resulted in a rapid and transient depolarisation with a slower time course than the excitatory junction potential produced by nerve stimulation. The iontophoretic application of DL-APB from the adjacent barrel to junctional glutamate receptors produced no detect-

Fig. 2 Time course of the inhibitory action of DL-APB on the junctional response to glutamate. The drugs are applied iontophoretically to junctional glutamate receptors from adjacent barrels of a twin-barrelled micropipette. Five superimposed traces are shown and on each, a single pulse of DL-APB precedes a smaller pulse of glutamate. As the interval between the DL-APB pulse and the glutamate pulse is increased, the junctional response to glutamate increases, that is, the inhibitory action of DL-APB decreases. The current passing through each barrel of the micropipette is monitored on the lower trace. 15 nC calibration refers to DL-APB which is the first pulse on the lower trace.



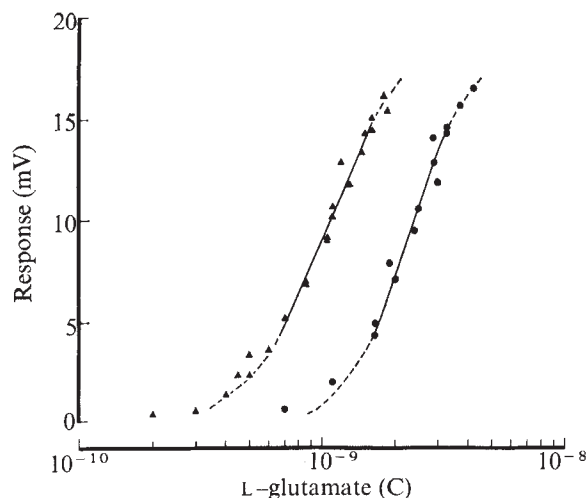


Fig. 3 Log dose-response curves illustrating the effect of locally applied glutamate alone (▲) and in the presence of DL-APB (●). The drugs were applied iontophoretically to junctional glutamate receptors from adjacent barrels of a twin-barrelled micropipette. In the right-hand curve, pulses of glutamate were applied in the presence of a constant pulse of DL-APB. Ordinate, amplitude of the junctional depolarising response to glutamate; abscissa, logarithmic scale of current (in C) passing through the glutamate barrel of the micropipette.

able response. When a pulse of DL-APB was applied to the junction during a steady glutamate application, however, the glutamate depolarisation was rapidly reduced and then recovered to the original depolarised level.

In most experiments a single pulse (40 ms) of DL-APB was followed by a single pulse (10 ms) of glutamate. Sufficient time was then allowed for complete recovery of receptors before again applying a single pulse of each drug. This precaution eliminated possible desensitisation effects. When glutamate application followed DL-APB application by a sufficiently short interval, the response to glutamate was absent or greatly diminished (Fig. 2). To study the time course of action of DL-APB the interval between single pulses of the two drugs was varied. The time constant of decay of DL-APB action (~400–500 ms) is approximately one third that of (+)-tubocurarine action at the frog end-plate¹⁶. The difference in time constants may represent a difference in the dissociation rate of the drug receptor complexes or in the speed of diffusion of the drugs away from the receptors.

The degree of interaction between glutamate and DL-APB varied depending on the dose of the two drugs (measured in nC), so for a constant dose of DL-APB the inhibition of the response to glutamate became less as the glutamate dose was increased. This may not necessarily represent competition between the agonist and the antagonist, because the time constant of dissociation of DL-APB from receptors is much longer than the rapid rise of the glutamate depolarisation, during which time dissociation would need to occur. In addition, larger doses of glutamate can be expected to reach more distant receptors^{15,17,18} which may be less affected by DL-APB.

To determine whether these two drugs were interacting in a competitive or non-competitive manner, dose-response curves were obtained for glutamate both in the presence and absence of a constant DL-APB pulse (Fig. 3). Problems arose from rapid decay of DL-APB action and the need to have a constant amount of inhibitor present over a larger area than was reached by glutamate. These were overcome by applying a short glutamate pulse (10 ms) at a fixed time during a much longer DL-APB pulse (500 ms). The dose-response curves for glutamate in the presence of DL-APB showed a parallel shift to the right, consistent with the concept of a competitive antagonism between the DL-APB and glutamate molecules, for the glutamate

receptor, although the possibility of a postsynaptic action of the DL-APB molecule at a level other than the glutamate receptor, cannot be discounted.

The junctional response to iontophoretically applied glutamate was not markedly inhibited by the application of DL-APB (5×10^{-4} M) in the bathing medium. Similarly miniature excitatory junctional potentials, which result from spontaneous release of transmitter packets, were unaltered. The apparent difference in effectiveness when DL-APB is applied iontophoretically may well reflect a difference in the concentration of drug reaching the receptors. Previous studies, however, have also shown that relatively high concentrations of substances, which specifically block the postsynaptic action of glutamate, may be required to produce antagonism of glutamate^{3,4}.

Both the biochemical and electrophysiological findings support the suggestion that DL-APB acts as a glutamate antagonist at the insect myoneural junction. It is hoped that the drug will prove to be a useful tool in the investigation of the nature and mode of action of the postsynaptic glutamate receptor.

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Susceptibility of teratocarcinoma cells to adenovirus type 2

As mouse embryos develop they become susceptible to infection by the small DNA oncogenic viruses, simian virus 40 and polyoma^{1,2}. Recently, the mouse teratocarcinoma, an alternative model system for the study of early development^{3,4}, was used in an approach to understanding how differentiation affects host-virus interaction (ref. 5 and M.B. and F.K., unpublished). Several differential teratocarcinoma lines were shown to be fully permissive to polyoma virus and could be transformed by SV40, therefore reacting to viral infection like most commonly used mouse cells. In contrast, the multipotential embryonal carcinoma cells were resistant to both viruses and the block seems to lie early in the lytic cycle, after penetration of the virus, but before synthesis of

the viral T antigen. Adenoviruses have much in common with the papovaviruses but are larger and more complex. We therefore examined the susceptibility of teratocarcinoma cells to these viruses. Here we report that both embryonal carcinoma cells and cells from a differentiated teratocarcinoma line are susceptible to adenovirus type 2.

The two teratocarcinoma cell lines used in this study have been derived from the transplantable mouse teratocarcinoma OTT 60-50-970 isolated by Stevens⁶. PCC3 (clone A/1) is a primitive embryonal carcinoma line which undergoes differentiation when injected into mice as well as *in vitro*^{7,8}. PCD3 was derived from embryoid bodies which were allowed to differentiate *in vitro*: it has a stable fibroblast morphology in culture and is non-tumorigenic (H. Jakob, personal communication). HeLa cells were used as a susceptible control. Following infection of PCC3 and PCD3 cells by adenovirus type 2, we screened the cells for production of viral antigens and infectious viral particles, as described in Table 1.

In brief, adenovirus antigens were detected by indirect immunofluorescence on cells seeded on glass coverslips, 20 h and 36 h after infection. The specific reagents were hamster anti-T antigen antiserum and rabbit multivalent capsid antiserum. Normal non-immune sera were used as controls. The indicator was fluorescein-conjugated anti- γ globulins (Institut Pasteur).

Virus titres were determined by the formation of foci on layers of HeLa cells⁹. These foci were stained by indirect immunofluorescence, with the use of anti-capsid serum as the specific reagent.

The results are summarised in Table 1. Synthesis of

Fig. 1 T-antigen immunofluorescence (indirect test) in Ad2-infected (a) multipotential PCC3/A/1 cells, and (b) differentiated PCD3 cells (see legend Table 1).

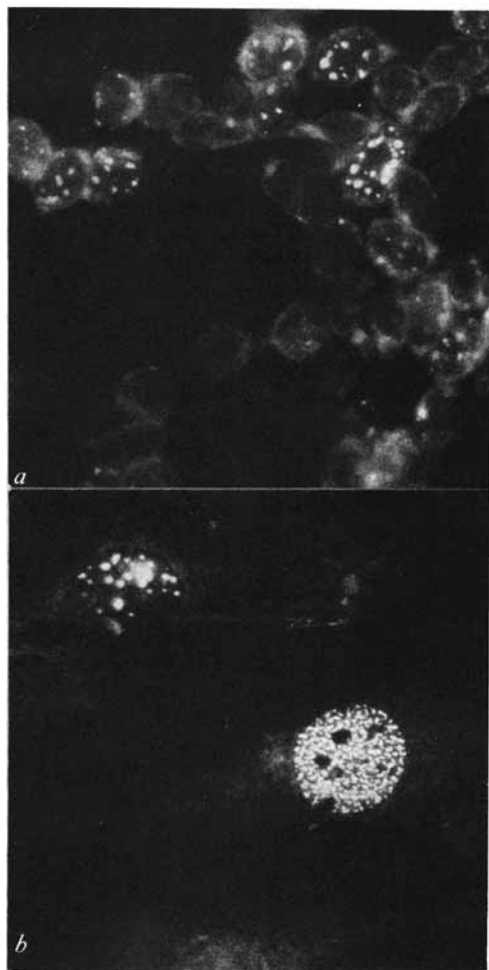


Table 1 Ad 2 antigens and virus production in HeLa cells and in two teratocarcinoma cell lines

Cells	T antigen*	Capsid antigen†	FFU ml ⁻¹ ‡
HeLa	50	100	1.0 10 ⁸
PCC3/A/1	40	16	1.7 10 ⁶
PCD3	38	53	0.9 10 ⁶

For detection of viral antigens, cells were seeded on glass coverslips (2×10^5 cells per 35-mm plastic Petri dish) and grown for 24 h at 37 °C in Dulbecco's modified Eagle's medium containing 10% foetal calf serum and in an atmosphere containing 12% CO₂. Cells were infected with adenovirus type 2 (a gift from Pierre Boulanger, INSERM, Lille) at an input multiplicity of 10 plaque-forming units per cell. Virus was adsorbed for 1 h at 37 °C. Cells were then covered with medium containing 2% foetal calf serum. Coverslips were removed 20 h and 36 h after infection, washed three times in phosphate-buffered saline and fixed in methanol-acetone (3 : 7) at -20 °C for 5 min. Adenovirus-specific proteins were detected by indirect immunofluorescence. The specific reagents were hamster anti-T-antigen antiserum (a gift from Pierre Boulanger, originally from Dr R. V. Gilden) and rabbit multivalent capsid antiserum (Pierre Boulanger). The indicator was fluorescein-conjugated anti- γ globulins (Institut Pasteur). *% cells positive 20 h after infection; †% cells positive 36 h after infection. Virus production was determined by fluorescent focus assay on HeLa cells⁹. Cells were grown and infected in the conditions described above on 60-mm dishes. Cells and overlying medium were collected 48 h after infection and frozen and thawed 3 times. HeLa cells grown on coverslips were infected with convenient dilutions of the cell extracts. They were fixed 48 h after infection and stained by indirect immuno-fluorescence using rabbit multivalent capsid antiserum, as the specific reagent. ‡Virus yield expressed as fluorescent focus units (FFU).

adenovirus-specific T antigen and of late capsid proteins was found to occur in both the primitive PCC3 and the differentiated PCD3 cells. The fluorescence pattern of the T antigen is characteristic of adenovirus, as shown in Fig. 1. Both types of cell produced infectious virus, although at a titre 50–100 times lower than HeLa cells.

These results show clearly that adenovirus type 2 can infect the primitive teratocarcinoma cells as efficiently as it can the differentiated cells. In contrast, the primitive cells are resistant to both polyoma virus and SV40: less than 1 in 10⁴ cells was T-antigen and V-antigen positive after polyoma infection and no T-antigen positive cell could be detected after SV40 infection (M. Boccarda and F. Kelly, unpublished). The diversity in response to the viruses is interesting in view of both similarities and differences exhibited by papovaviruses and adenoviruses. All three viruses replicate exclusively in the nuclei of the permissive cells and the viral genome is integrated into the host chromosome during the lytic cycle¹⁰. Adenoviruses, however, contain seven times as much DNA and are also more complex. In particular, at least two early genes of adenovirus are necessary for replication of the viral DNA¹¹, whereas early mutants of SV40 and polyoma fall into only one complementation group. It is also interesting to note that adenovirus recombines far more efficiently than does SV40 or polyoma.

Because very little is known about the early events which take place in the nucleus before initiation of viral transcription and the appearance of the T antigen, one can only speculate on the basis for the different response of the primitive stem cells to papovaviruses and adenoviruses. The hypothesis we favour is that one or several cellular functions required early in the lytic cycle of SV40 and polyoma, possibly recombination between host and viral genomes, is missing in these cells. A function, carried or coded by the more complex adenoviruses could obviate this deficiency. Experiments are in progress to test whether coinfection by adenovirus will help SV40 or polyoma to infect primitive teratocarcinoma cells. We are also attempting to obtain clones of primitive cells containing a fragment of the adenoviral genome, which may be useful as a marker during their differentiation.

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Is the scrapie agent a virus?

THE nature of the scrapie agent has been obscure for many years. It is not known whether it is a virus in a strict sense, some replicating agent which is devoid of nucleic acid^{1,2}, or a transmissible self-replicating cellular membrane^{3,4}. In previous experiments with the Chandler strain of the scrapie agent, numerous 14-nm virus-like particles were isolated⁵ from infected mouse brains using repeated fluorocarbon extraction procedures which have been used for the isolation of Aleutian disease virus from infected mink tissues^{6,7}. The present experiments were designed; first, to determine if similar 14-nm virus-like particles could be isolated from hamster brains infected with scrapie agent; and second, to determine if the 14-nm virus-like particles induce scrapie in mice and hamsters.

Hamster brains (39 g) infected with the scrapie agent were obtained from Dr R. F. Marsh⁸ of the University of Wisconsin. The hamster brain suspensions were extracted repeatedly with fluorocarbon as described previously⁵. The final scrapie extracts from the 39 g of hamster scrapie brains were resuspended in 1.5 ml of 0.05 M Tris-HCl, pH 7.4. A CsCl density gradient ultracentrifugation was performed with these hamster scrapie extracts at 25 °C at 243,000g for 72 h. In the same run, three other preparations (mouse scrapie brain extracts, normal mouse brain extracts and normal hamster brain extracts) were also ultracentrifuged. Mouse scrapie brain extracts and normal mouse brain extracts were used as prepared previously⁵. Normal hamster brain extracts were prepared from the same strain of 10-week-old hamsters (LVG: LAK strain) (purchased from Lakeview Farms, Newfield, New Jersey). From 68 g of normal hamster brains, 1.7 ml of normal hamster brain extract was prepared. After the CsCl gradient ultracentrifugation, only one band was visible with the normal mouse brain and normal hamster brain extracts. This band was located in the region of buoyant density of 1.29–1.30 g cm⁻³ in the CsCl (fraction 5). With the mouse and hamster scrapie extracts, however, one band was observed at the same place and another in the region of buoyant density of 1.33–1.34 g cm⁻³ (fraction 6). Numerous virus-like particles, measuring about 14–15 nm in diameter as seen previously⁵ were observed in the second band of both hamster and mouse scrapie extracts. The structure and size of these particles from these two scrapie extracts from mice and hamsters are identical. No virus-like particles were observed in the first band (density 1.29–1.30 g cm⁻³) of any of the four different preparations, nor in the regions

of buoyant density of 1.33–1.34 g cm⁻³ of normal hamster or mouse brain extracts. The CsCl gradient was collected in 10 fractions from the top of the tube using Pasteur pipettes. Particles with the structure of membranes were not detected in the fractions from the four different extracts.

Comparative titres of infectivity of the different stages of fluorocarbon extractions and the CsCl gradient fractions of mouse scrapie extracts were determined in mice. Groups of six to ten Swiss albino mice were inoculated intracerebrally with 0.03 ml in serial tenfold dilutions of the various scrapie preparations. Each experiment of mouse scrapie infectivity was terminated 47 weeks after inoculation and brains and spinal cords removed from those mice which developed scrapie symptoms. Scrapie was diagnosed by the astrocyte response in these tissues using the Cajal's gold sublimate method as described by Hadlow⁹. The infective dose (ID₅₀) defined as that dilution of material which induced scrapie in 50% of the inoculated animals, was calculated by the method of Reed and Muench¹⁰.

The results of titration of mouse scrapie preparations are shown in Table 1. Scrapie infectivity was markedly reduced by the repeated fluorocarbon extraction procedures and CsCl gradient ultracentrifugation. In the CsCl gradient of mouse scrapie extracts, maximum infectivity was detected in the pooled fractions 6–8 which yielded 14-nm virus-like particles. In this group of mice, scrapie symptoms were first observed 23 weeks after inoculation, whereas in those mice inoculated with mouse scrapie brain suspension, scrapie symptoms were first observed at 17–18 weeks after infection. The astrocyte response was always detected in mice which developed scrapie symptoms.

Infectivity of hamster scrapie brain (195 ml), final hamster scrapie extracts (1.5 ml) and of the pool of fractions 6–7 (1.5 ml) of the CsCl gradient of hamster scrapie extracts which yielded the 14-nm virus-like particles, was titrated in hamsters by inoculation with 0.05 ml intracerebrally. The infectivity titres of these three inocula were ID₅₀ 10^{8.7}, 10^{5.5} and 10^{3.5} per 0.05 ml respectively during the observation period of 39 weeks. The diagnosis of scrapie in hamsters was also confirmed by astrocyte response. In hamsters, scrapie symptoms were first observed 10 weeks after infection with hamster scrapie brain suspension and after 12 weeks with the other two preparations. The infectivity titre of hamster scrapie extracts is being determined more precisely using fractions 1–5 collected from the top of the CsCl gradient, and fractions 6–10 through a hole in the bottom of the tube.

Previous studies⁵ showed that the final mouse scrapie extract from 132 g of brain contained 8.8 mg of protein in a total volume of 10 ml. Therefore, the fluorocarbon stage in the purification of virus-like particles leads to the removal of about 99.99% or “4 logs” of brain tissues. In the present experiments 5.8 log₁₀ID₅₀ units of mouse infectivity and 5.2 log₁₀ID₅₀ units of hamster infectivity were lost during fluorocarbon treatment, and exposure to CsCl caused further decreases in the infective titre. This marked reduction of scrapie infectivity is consistent with the observations of others¹¹ that the scrapie agent is inactivated by various lyophilic solvents and by CsCl. The density of the 14-nm particles and the maximum infectivity found in fractions 6–8, is however, consistent with the report of Gibbs¹² in which maximum scrapie infectivity was observed at densities between 1.32 and 1.37 g cm⁻³ in a CsCl gradient.

In view of the findings that identical 14-nm virus-like particles were isolated from the brains of two different animal species (mouse and hamster) obtained from two different laboratories, scrapie infectivity was detected in the fractions yielding these 14-nm particles, and as these virus-like particles form a homogenous band in a CsCl gradient, I suggest that the 14-nm particles are likely to be the causative agent of scrapie.

I thank W. J. Hadlow for providing mouse scrapie brains

Table 1 Infectivity of mouse scrapie brain extracts

Preparations		Amount (ml)	Titres of scrapie infectivity	
			$\log_{10}ID_{50}$ per 0.03 ml	Total titres ($\log_{10}ID_{50}$)
Scrapie mouse brains suspension		790	7.0	11.4
↓ Freon 113 extraction				
↓ 10,000 <i>g</i> 30 min supernatant		660	4.9	9.2
↓ 100,000 <i>g</i> 5 h supernatant		660	2.0	6.3
↓ 100,000 <i>g</i> 5 h pellets		100	Not done	—
↓ Freon 113 extraction				
↓ 10,000 <i>g</i> 30 min supernatant		100	3.8	7.3
↓ 100,000 <i>g</i> 10 h supernatant		100	1.4	4.9
↓ 100,000 <i>g</i> 10 h pellets (mouse scrapie extracts)		10	3.1	5.6
CsCl fractions*	Electron microscopy	Total amount		
1-4 pool	No virus	10†	≤0.5	≤3.0
5	No virus	10	≤0.58‡	≤3.08
6-8 pool	Numerous 14-nm virus-like particles and some ferritins	10	2.5	5.0
9-10 pool	Ferritins	10	≤0.5	≤3.0

* Fractions were collected from top of the table. Two bands were visible: in fraction 5 (density 1.29–1.30 g cm⁻³) and in fraction 6 (density 1.33–1.34 g cm⁻³).

† Only 1 ml of the total amount of 10 ml mouse scrapie extract was centrifuged on CsCl. The CsCl fractions were diluted with 0.85% saline, concentrated by centrifugation at 300,000g for 5 h and the pellets were suspended in 1 ml of saline.

‡ Scrapie was diagnosed in one of 7 inoculated at 10⁻¹ dilution at 35 weeks. No mice showed scrapie symptoms at 10⁻¹ or higher dilutions in the fractions 1-4 and 9-10 pools.

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Rifampicin-resistant RNA polymerase and NAD transferase activities in coliphage N4 virions

SEVERAL animal viruses carry within the infectious particle a number of enzymatic activities^{1,2}. Prominent among these are the RNA synthetases of orthomixoviruses, paramixoviruses, rhabdoviruses³⁻⁶, the DNA-dependent RNA polymerase of poxviruses⁷ and the reverse transcriptases of oncoviruses^{8,9}, all of which play important roles in the virus life cycle. Exclusion of vital enzymes from the virion of all complex bacterial viruses seems, on the other hand, to have been the result of the evolutionary trend followed by these agents. A notable exception to this rule seems to

be represented by coliphage N4 (ref. 10). It has been reported that transcription of N4 early genes^{11,12} and incorporation of ³²P-orthophosphate into several host proteins¹³ occur in *Escherichia coli* cells treated before infection with rifampicin and chloramphenicol. A possible explanation for these findings was that N4 injects into the cell the relevant enzymes along with its DNA. We report here the detection of a rifampicin-resistant RNA polymerase and a nicotinamide adenine dinucleotide (NAD) transferase in purified N4 particles.

To demonstrate the existence of virion-associated enzymes several procedures were successfully developed for the rupture of the virus protein coat¹⁴ since preliminary experiments failed to show any activity in intact phage preparations. Disruption of isopycnically purified N4 (ref. 10) by three cycles of freezing in liquid nitrogen and thawing at 37 °C was finally adopted for the sake of simplicity. These N4 cryolysates were briefly sonicated and the DNA precipitated with streptomycin. Residual intact virions, ghosts and DNA were removed by centrifugation, and the clear extracts, after dialysis, served as the source of enzyme.

Table 1 shows that incorporation of ³H-UTP into RNA was mediated by these N4 preparations in a modified RNA polymerase assay¹⁵. Like the RNA polymerase of *E. coli*, N4 enzyme requires all four ribonucleotide triphosphates, Mg²⁺ and a DNA template for optimal activity. The acid-insoluble product is totally susceptible to RNase digestion, and it is not synthesised in the presence of actinomycin D. On the other hand, N4 enzyme is inhibited by low concentrations of monovalent ions and by phosphates, and shows a stringent template specificity in that only homologous phage DNA acts as an efficient primer. Furthermore, the functioning of N4 polymerase is highly resistant to the antibiotic rifampicin at concentrations that inhibit *E. coli* RNA polymerase^{16,17}.

As an indication of the fidelity of transcription by the phage enzyme, RNA synthesised *in vitro* from N4 DNA hybridised with high efficiency (43%) to denatured N4 DNA

Table 1 Characteristics of N4 virion-associated RNA polymerase activity

	Activity (%)
Complete system	100
Omit ATP, or CTP, or GTP	1
Omit Mg ²⁺	8
Omit N4 DNA	2
Add calf thymus DNA (50 µg ml ⁻¹)	18
Add <i>E. coli</i> DNA (200 µg ml ⁻¹)	7
Add 0.15 M KCl	21
Add 0.05 M K phosphate, pH 7.0	19
Add RNase (10 µg ml ⁻¹)	1
Add actinomycin D (10 µg ml ⁻¹)	1
Add rifampicin (5 µg ml ⁻¹)	96

Isopycally purified N4 preparations were obtained as described previously³⁰. After three cycles of freezing in liquid nitrogen and thawing at 37 °C the viscous lysates were sonicated for 30 s in a Branson apparatus at an output of 50 W. Removal of DNA was effected by adding streptomycin to a final concentration of 6%. After 15 min at 4 °C, precipitated DNA, ghosts and residual intact phage particles were sedimented by spinning at 40,000 r.p.m. for 90 min in the 50 rotor of an L3-50 Spinco ultracentrifuge. After thorough dialysis against 0.01 M Tris-HCl buffer, pH 7.2, containing 0.001 M MgCl₂, the clear extract was used as the source of enzyme.

Complete system for the assay of N4 polymerase activity contained 10 µmol Tris-HCl (pH 7.9), 2.5 µmol MgCl₂, 25 nmol dithiothreitol, 25 nmol EDTA, 125 µg bovine serum albumin, 2 pmol N4 DNA, 37.5 nmol each of the four ribonucleoside triphosphates of which UTP was labelled with ³H (Amersham, England; specific activity 5 × 10³–2 × 10⁴ c.p.m. nmol⁻¹) and 30 µl N4 cryolysate in a final volume of 250 µl. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of 3 ml ice-cold 5% TCA containing 10⁻² M sodium pyrophosphate. The acid-insoluble RNA product was collected on Whatman GF/C filters, washed and counted as reported previously³⁰. For the complete system 100% activity was 5,200 c.p.m.

(Table 2). In addition, unlabelled RNA synthesised by N4-infected Hfr 3300 cells either untreated or pretreated with rifampicin and chloramphenicol^{11,12} competed (greater than 90% inhibition) with labelled RNA produced *in vitro*. These data indicate that the RNA species synthesised *in vitro* by N4 virion polymerase are not qualitatively different from those produced during *in vivo* infection.

Phosphorylation of proteins is catalysed by phage-coded products through at least two mechanisms involving either protein kinases that transfer phosphate from ATP to seryl groups of acceptor molecules¹⁸, or NAD transferases that modify target polypeptides by the simultaneous addition of adenosine and phosphorus^{19–21}. None of these enzymes has been shown to be virion associated.

N4 particles do not carry a protein kinase since ³²P was not transferred to proteins in reaction mixtures containing γ-³²P-ATP (not shown). On the other hand, N4 cryolysates were active in transferring radioactivity from ¹⁴C-NAD into acid-precipitable linkage with various acceptor proteins (Table 3). Among those proteins tested, lysozyme was the best acceptor. Dithiothreitol markedly stimulated ¹⁴C-NAD incorporation possibly implying an activation of the enzyme by thiol compounds²¹.

The label bound to the reaction product resisted heat treatment in mild acidic and alkaline conditions, and digestion with DNase. After treatment with Pronase and snake venom phosphodiesterase ¹⁴C-radioactivity was converted to an acid-soluble form (not shown). These results suggest that N4 enzyme may be similar to the ADP-ribosyl transferase activities that have been established for diphtheria toxin^{21,22}, *Pseudomonas aeruginosa* PA toxin²³ and T4 'alteration' factor²⁰.

In a preliminary effort to characterise the molecular nature of N4 enzymes, ³⁵SO₄-labelled isopycally purified virions were disrupted, treated with DNase, and clarified from ghosts and residual intact particles by centrifugation; the supernatant containing both enzymatic activities was electrophoresed in sodium dodecyl sulphate (SDS)-polyacrylamide slab gels²⁴. In the autoradiogram shown in Fig. 1 two radioactive bands with approximate molecular weight



Fig. 1 Identification of polypeptides present in N4 virion cryolysates. Isopycally purified ³⁵SO₄-labelled N4 particles³⁰, disrupted by three cycles of freezing and thawing, were treated with DNase (5 µg ml⁻¹) for 15 min at 37 °C. Ghosts and residual intact virions were removed by spinning as specified in Table 1. Samples of intact radioactive particles (25 µl, 50,000 c.p.m.) and enzymatically active cryolysates (25 µl, 1,100 c.p.m.) were prepared for SDS-polyacrylamide electrophoresis in 10% slab gels using the procedures and apparatus described previously^{24,34}. In the autoradiogram shown the origin of electrophoresis is at the top. *a*, Migration pattern of N4 cryolysates; *b*, migration pattern of intact N4 virion polypeptides.

of 65,000 are visible. Whether these polypeptides can be considered dissociated subunits of one or both N4 enzymes or merely represent internal proteins, remains to be established.

Although rifampicin-resistant phage-coded RNA polymerases are known to be synthesised in infected cells during the life cycle of T7 (ref. 25), T3 (ref. 26), PBS2 (ref. 27) and gh-1 (ref. 28) bacteriophages, N4 enzyme provides the first example of a virion-associated transcription apparatus in bacterial viruses.

The occurrence of this enzyme as an integral part of N4 may be of evolutionary significance for a virus which replicates exclusively in the polar regions of its host²⁹. Besides,

Table 2 Hybridisation-competition of N4 RNA polymerase product by various RNA preparations

Type of non-radioactive competing RNA	Amount of <i>in vitro</i> RNA (c.p.m.)	c.p.m. in hybrid	% RNA hybridised
None	3.1 × 10 ³	1.3 × 10 ³	43
Uninfected <i>E. coli</i> RNA (200 µg ml ⁻¹)	3.1 × 10 ³	1.3 × 10 ³	43
RNA isolated from <i>E. coli</i> cells 4 min after N4 infection (50 µg ml ⁻¹)	3.1 × 10 ³	1.2 × 10 ³	3.9
RNA isolated from <i>E. coli</i> cells pretreated with rifampicin and chloramphenicol, and infected for 4 min with N4 (50 µg ml ⁻¹)	3.1 × 10 ³	1.1 × 10 ³	3.5

Labelled RNA synthesised by N4 virion-associated RNA polymerase in the conditions reported in Table 1, was purified from the reaction mixture by phenol extraction¹¹. Competitor RNA molecules were prepared from uninfected or N4-infected *E. coli* Hfr 3300 cells as specified previously¹¹. Conditions of N4 infection in the presence of chloramphenicol and rifampicin have been described¹². RNA-DNA hybridisation was carried out as already reported¹¹ using N4 DNA (2 µg) bound to nitrocellulose filters.

Table 3 Characteristics of N4 virion-associated NAD transferase activity

	Activity (%)
Complete system	100
Omit dithiothreitol	7
Omit Mg ²⁺	52
Substitute lysozyme with the following acceptor proteins:	
Histones	63
<i>E. coli</i> cell-free extract	56
RNase	30
Protamine	9
Bovine serum albumin	7
N4 ghosts	6
Casein	5

N4 cryolysates were obtained by freezing and thawing as specified in Table 1. The viscous lysates were treated with DNase (3 µg ml⁻¹) at 37 °C for 15 min. Ghosts and residual intact virions were removed by centrifugation at 40,000 r.p.m. for 90 min in the 50 rotor of an L3-50 Spinco Ultracentrifuge and the clear extract was used as the source of enzyme.

Complete system for the assay of N4 NAD transferase activity contained 4 µmol Tris-HCl (pH 8.2), 7.5 nmol EDTA, 3 µmol dithiothreitol, 4 µmol MgCl₂, 30 µg lysozyme (Koch and Light), 50 pmol [¹⁴C]-NAD (Amersham, England; specific activity 280 Ci mol⁻¹) and 15 µl N4 cryolysate in a total volume of 65 µl. RNase (Boehringer) bovine serum albumin (Sigma), histones (Sigma, fraction II), Protamine (Sigma) and Casein (Sigma), when used as acceptors, were present in the reaction mixture at a concentration of 30 µg. Hfr 3300 cells grown in 50 ml M9S medium²⁹ at a concentration of 5 × 10⁸ ml⁻¹ were collected by centrifugation, washed in distilled water, resuspended in 1 ml 0.001 M Tris-HCl buffer (pH 8.2) and sonicated at 0 °C for 2 min in a Branson sonifier at an output of 70 W. After centrifugation at 10,000 r.p.m. for 10 min, 10 µl of the clear cell-free extract were added to the NAD transferase reaction. Purified N4 ghosts prepared as reported previously¹⁴ were added at a final concentration of 20 µg. After incubation at 25 °C for 60 min the reaction was terminated by addition of 65 µl 10% TCA. The acid-insoluble radioactivity was collected on Whatman GF/C filters, washed with 5% TCA and counted as detailed previously³⁰. For the complete system 100% activity was 1,850 c.p.m.

the identification of a rifampicin-resistant RNA polymerase in N4 particles provides a likely explanation for the drug resistance of phage transcription during the early stages of replication^{11,12}. This enzyme is injected along with the phage genome at infection. Once in the cell the injected polymerase will immediately direct the synthesis of additional drug-resistant RNA polymerase molecules by transcribing N4 cistrons 3 and 4 (ref. 30) which probably code for this activity. Expression of the early genes then takes place at a suitable rate and the phage DNA is replicated^{11,12}. Late genes are next transcribed with the mandatory participation of an N4-modified form of *E. coli* polymerase³¹. During morphogenesis N4-coded RNA polymerase or a catalytically active subassembly of this enzyme is finally packaged into the infectious virions. Mero complex models of N4 gene expression can also be constructed on the basis of present and previous evidence.

When injected into the host, N4-associated NAD transferase catalyses the phosphorylation of about 30 bacterial proteins, which do not include any of the host RNA polymerase subunits¹³. A NAD transferase which 'alters' the α subunit of *E. coli* RNA polymerase^{19,32} has been tentatively located by Horvitz³³ in T4 particles. T4 mutants deficient in 'alteration' have been shown to be perfectly viable³³. It has been suggested that the main role of 'alteration' may reside in the ability to confer a broad host range on T4 phage³³. Since N4 replicates productively only in the K12 strains of *E. coli*³⁰, amplification of the host range by possession of the virion-associated NAD transferase seems improbable. Considering the scarcity of receptors for N4 on the surface of sensitive cells²⁹, we favour the hypothesis that during evolution N4 NAD transferase was retained in the virion because of some positive role it may have in initiating the infectious process. Detailed physicochemical and biochemical characterisation of N4 virion-associated enzymes will be reported elsewhere.

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Human cytomegalovirus infection of WI-38 cells stimulates mitochondrial DNA synthesis

HUMAN cytomegalovirus (HCMV) infection has been shown to lead to the stimulation of host cell DNA and RNA synthesis in both permissive and non-permissive cells¹⁻³. This effect on DNA synthesis occurred at about 12-16 h after infection, and preceded synthesis of viral DNA by 36 h. The host cell DNA species which are stimulated have not been characterised. We show here that much of the stimulation of DNA synthesis was due to an increased rate of mitochondrial DNA synthesis and that ethidium bromide inhibited HCMV replication.

Confluent cultures of WI-38 cells were infected with HCMV at a multiplicity of infection of 1 plaque-forming unit (PFU) per cell. In all the experiments (unless otherwise noted) WI-38 cells were infected at least 5 d after reaching confluency in minimum essential medium (MEM) plus 10% foetal bovine serum. After infection the cultures were refed with MEM containing 0.4% foetal bovine serum, to minimise the stimulatory effect of serum on cellular DNA, and incubated at 37 °C. In these conditions, typical nuclear inclusion bodies for HCMV were demonstrated in virtually all of the cells 3 d after infection. Total DNA synthesis was measured by labelling the cells for 60 min with [³H]-thymidine (³H-dT) at various times after infection. Mock-infected cultures were used as controls.

The incorporation of ³H-dT into cellular DNA and the HCMV infectivity of WI-38 cells at various intervals are shown in Fig. 1. Infectivity was not seen until 48 h after

infection, when a small amount of virus was detected; this amount subsequently increased.

Apparently the incorporation of ^3H -dT occurred in two phases: the first reaching a maximum at 16 h after infection, then decreasing until 36 h, when the second phase begins. Previous DNA analysis by equilibrium CsCl centrifugation showed viral DNA only in specimens obtained 36 h after infection (ref. 1) and reached a peak at 96 h.

Mitochondrial DNA was isolated from cytoplasmic extracts by zonal centrifugation in sucrose density gradients and then analysed in equilibrium CsCl -ethidium bromide gradients. Figure 2 presents the incorporation of ^3H -dT into the mitochondrial DNA of WI-38 cells labelled for 1 h at 16 h after infection. Most of the radioactive precursor was incorporated into mitochondrial DNA. The rate of ^3H -dT incorporation into mitochondrial DNA in infected cells was approximately five times that in control cells. As expected⁴, the greater part of the incorporated radioactivity was found in the same fraction as the closed circular SV40 DNA added to the gradient as a marker.

Analysis of mitochondrial DNA at various times after infection revealed that the rate of incorporation of ^3H -dT into mitochondrial DNA of infected cells was always 1.5 to 5 times higher than in the control cells and the greatest increase occurred between 12 and 18 h after infection. Mitochondrial DNA labelled with ^{14}C -dT and prepared from mock-infected cells by the same type of CsCl gradient procedure was then mixed with SV40 DNA as marker, and the mixture sedimented through a 5–20% sucrose gradient at neutral pH. The peaks of ^3H -dT label from infected cells coincided with the peaks of the ^{14}C label from mock-infected cell DNA (Fig. 3).

Greater incorporation of a DNA precursor into mitochondrial DNA is insufficient as evidence of an increase in the rate of synthesis of mitochondrial DNA in the infected cells, because the pool size of thymidine, the kinase levels and the rate of turnover may differ from those in uninfected cells. For these reasons, additional experiments were carried out at 12 and 24 h past infection. Cultures prelabelled with ^{32}P were used to measure specificity of the acid-soluble pool of dT triphosphate. Inorganic $^{32}\text{PO}_4$ ($5 \mu\text{Ci ml}^{-1}$) was added to the culture and the cells were continuously labelled for 48 h before infection. ^3H -dT ($10 \mu\text{Ci ml}^{-1}$) was added to infected and non-infected cells for 1 h at 12 h and 24 h after infection. The ratio of ^{32}P to ^3H in dT triphosphate

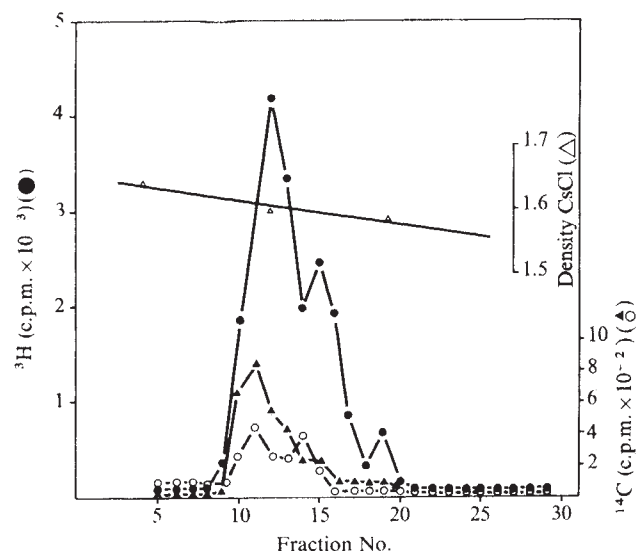
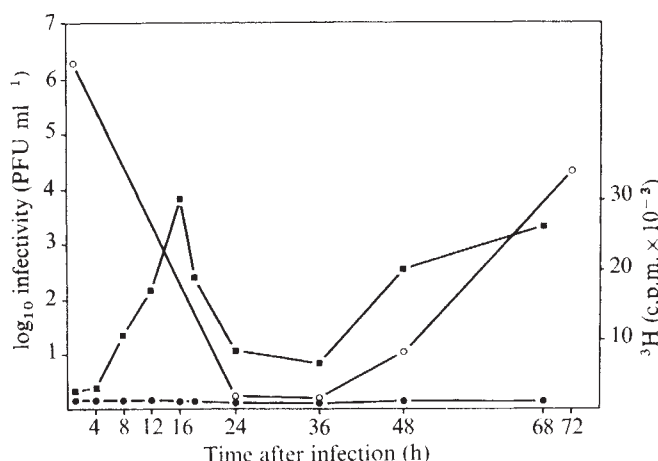


Fig. 2 WI-38 cells (3×10^6) were infected, 5 d after reaching confluent monolayers, with HCMV at a multiplicity of 1 PFU per cell. At 16 h after infection, cells were labelled for 1 h with $5 \mu\text{Ci ml}^{-1}$ of ^3H -dT (specific activity 6.7 Ci mmol^{-1}). Mock-infected cells were treated identically. At the end of the incubation cytoplasmic extracts were prepared according to a method previously described¹⁴, and were layered on to 15–30% sucrose-sodium dodecylsulphate gradients and centrifuged at 25,000 r.p.m. in an SW27 rotor for 16 h at 25°C . Fractions were collected from the gradient. The fractions corresponding to the lower half of the gradient were pooled and precipitated with alcohol. Nucleic acids were resuspended in 10 mM Tris (pH 7.6) and 1 mM EDTA made 2 M in CsCl , and left overnight. After centrifugation at $15,000g$ for 10 min, the solution received CsCl to a density of 1.530 g cm^{-3} and ethidium bromide to a concentration of $250 \mu\text{g ml}^{-1}$. Centrifugation was carried out in the SW50 Spinco rotor for 36 h at 44,000 r.p.m. at 20°C . The position of supercoiled DNA molecules was checked using SV40 circular DNA labelled with ^{14}C as a marker. ●, Infected; ○, mock-infected; ▲, SV40 circular DNA.

Fig. 1 Monolayers of WI-38 cells in 3-cm Petri dishes containing 3×10^5 cells were infected at an input multiplicity of 1. At various times after infection, ^3H -dT was added at a concentration of $1 \mu\text{Ci ml}^{-1}$ and the cells incubated at 37°C for 60 min. After incubation the cells were lysed with 0.1% sodium dodecylsulphate and precipitated with 5% trichloroacetic acid. Precipitates were filtered and radioactivity counted. Infectivity of cell-associated virus was assayed after disrupting the cells by sonication¹³. ○, Infectivity; incorporation of ^3H -dT, ●, mock-infected cells; ■, infected cells.



was measured using the methods for acid-soluble extracts and polyethyleneimine thin-layer chromatography as described by Neuhaud *et al.*⁵. The specific activities of the acid-soluble pools of dT triphosphate were unaffected in both infected and uninfected cultures.

Because HCMV has a long eclipse period (48–72 h)^{6–8}, the stimulation of mitochondrial DNA occurs before viral replication. It was interesting, therefore, to determine the effect of ethidium bromide on both mitochondrial DNA and viral production. Confluent monolayers of WI-38 cells were infected with HCMV and after allowing 1 h for virus adsorption, medium containing various concentrations of ethidium bromide was added. At intervals after infection, the total incorporation of ^3H -dT into DNA was measured as in the experiment presented in Fig. 1. Infectivity of cell-associated virus was assayed 3 d after infection. At concentrations of 5 and $1 \mu\text{g ml}^{-1}$, ethidium bromide completely abolished the HCMV-induced DNA stimulation and decreased infectivity by more than three orders of magnitude. At a concentration of $0.1 \mu\text{g ml}^{-1}$ of ethidium bromide, incorporation of ^3H into DNA was only slightly decreased whereas infectivity was about ten times lower than in the untreated culture (Fig. 4).

The data presented here indicate that HCMV stimulated mitochondrial DNA in cells where DNA synthesis was repressed by contact inhibition or by depletion of serum⁹. The stimulation of mitochondrial DNA preceded viral DNA replication and was thus an expression of an early function of the viral genome. These results are not analogous to those observed in cells infected with simian virus 40 (SV40), polyoma or herpes simplex virus. SV40 and polyoma stimulate mitochondrial DNA synthesis concomitantly with virus-directed induction of nuclear DNA

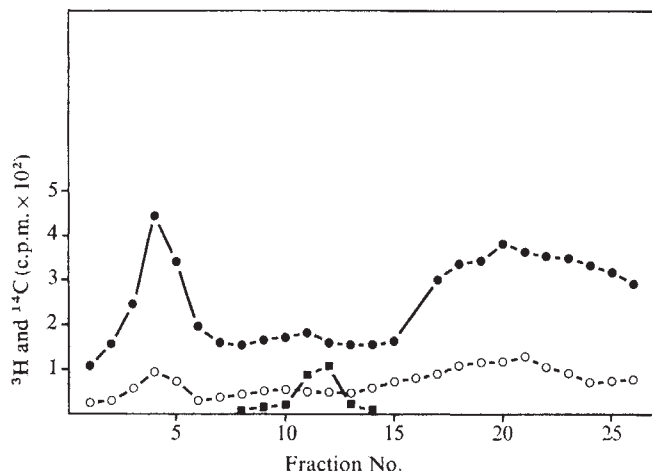


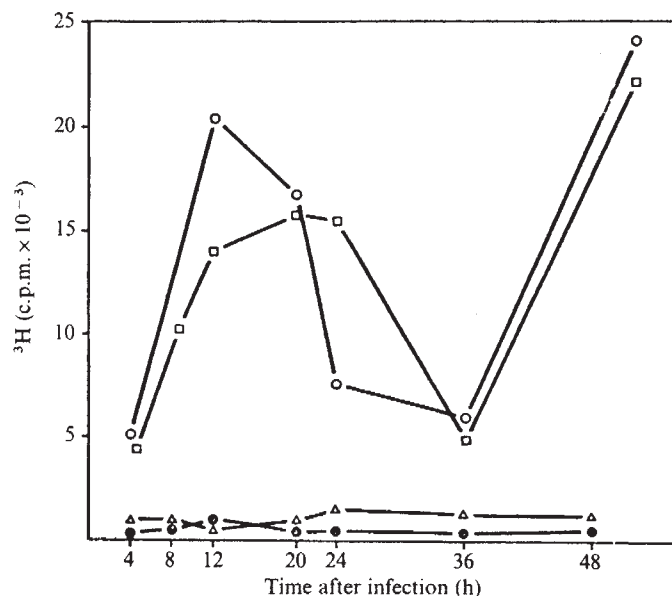
Fig. 3 DNAs from infected or mock-infected cells were first purified by centrifugation in CsCl-ethidium bromide gradients as described in Fig. 2. The two peaks from these gradients (fractions 12 and 15) were then dialysed and sedimented through a neutral 5–20% sucrose gradient for 10 h at 25,000 r.p.m. in an SW27 rotor. Fractions were collected as shown in the figure, and radioactivity measured. A sample of SV40 circular DNA was added to a gradient containing mock-infected cell DNA. ●, ^3H -DNA from infected cells; ○, ^{14}C -DNA from mock-infected cells; ■, SV40 DNA.

synthesis^{4,10,11}. In herpes simplex infection, nucleus-directed macromolecular synthesis is progressively inhibited, and mitochondrial stimulation may reflect the elimination of the nuclear repressor system for mitochondrial DNA replication¹¹.

That stimulation of mitochondrial DNA synthesis by HCMV is not mediated through a stimulation of nuclear DNA is indicated by the fact that the latter was demonstrated only late in the growth cycle of the virus. Further work is necessary to clarify the relationship between the stimulation of mitochondrial and nuclear DNA.

Although the biological significance of the inhibitory

Fig. 4 Confluent monolayers of WI-38 cells in Petri dishes (diameter 3 cm) were infected with HCMV. After 1 h absorption, the cells were incubated in media containing various concentrations of ethidium bromide. At the indicated times after infection, ^3H -dT was added at a concentration of $1 \mu\text{Ci ml}^{-1}$ for 30 min. Samples to measure radioactivity incorporated into the cells were processed in the same manner as that described in Fig. 1. Infectivity of cell-associated virus was assayed at 4 d after infection. ○, Without drug; □, $0.1 \mu\text{g}$ ethidium bromide; △, $1 \mu\text{g}$ ethidium bromide; ●, $5 \mu\text{g}$ ethidium bromide.



effect of ethidium bromide on the growth of HCMV must remain speculative, two possibilities can be considered. HCMV may require more energy from mitochondria for replication than do other viruses. Alternatively, since, at low concentrations, more ethidium bromide is bound to supercoiled DNAs than to normal double helices¹², the DNA of HCMV may form circular DNA at a certain stage of the replication cycle. In any case it is clear from the data that HCMV acts as a trigger for the stimulation of mitochondrial DNA synthesis and this stimulation may be a necessary factor for subsequent HCMV replication.

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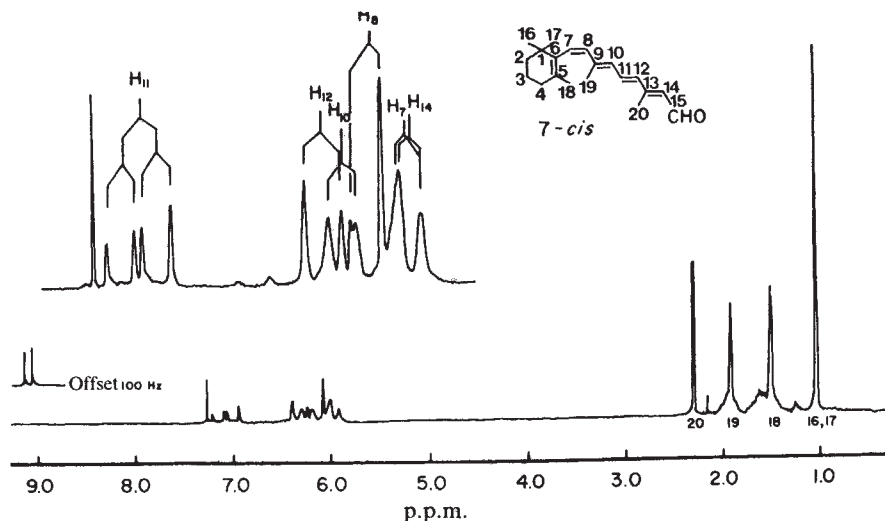
Rhodopsin analogues from highly hindered 7-*cis* isomers of retinal

ALL known visual pigments have as chromophore the sterically hindered 11-*cis* isomer of retinal. Shortly after this was established, an unsuccessful attempt was made to prepare the much more hindered 7-*cis*-retinal by partial reduction of the corresponding 7,8-acetylene¹. But, four new geometric isomers of retinal, all apparently 7-*cis*, have been synthesised by a six-step non-stereospecific sequence². Structural assignments of the isomers were originally based on spectral data of two fractions of partially separated mixtures of isomers (7-*cis* and 7,9-dicis; 7,13-dicis and 7,9,13-tricis). Their configurations were, therefore, not completely firm. Since then two of the four isomers (7-*cis* and 7,9-dicis) have been isolated by high pressure liquid chromatography (HPLC). Their nuclear magnetic resonance (NMR) spectra unambiguously confirmed the tentative assignments made earlier. That of 7-*cis*-retinal is shown in Fig. 1 as a representative example. The remaining two 13-*cis* isomers remain unseparated in various HPLC conditions. Their structures, however, have since been confirmed through a stereo-selective synthesis of 7,9,13-tricis-retinal³. We report here preliminary results of the interaction of these 7-*cis*-retinal isomers with cattle opsin.

The ultraviolet absorption spectra of 7-*cis*-, 7,9-dicis- and 7,9,13-tricis-retinal are shown in Fig. 2 together with that of all-*trans*-retinal. The relatively small hypsochromic shift of the α -band of the 7-*cis* isomers is consistent with the view of increased non-planarity only near the terminal positions of the polyene chain (that is, about the 6,7-bond) in 7-*cis* isomers⁴⁻⁷. Thus, the 7,9,13-tricis isomer has a higher molar absorbance than 11-*cis* (36,600 compared with 26,400 (ref. 8)).

All three isomers were tested with cattle opsin as to their possible formation of 7-*cis* analogues of rhodopsin. Cattle rod outer segment (ROS) membranes were isolated from frozen Hormel retina and stored in buffered solution according to the reported procedures^{9,10}. When incubated with opsin in suspen-

Fig. 1 Fourier-transform NMR spectrum of 7-*cis*-retinal in CDCl_3 recorded on a Varian XL-100 spectrometer. The inset shows an expansion of the region between $\delta 6$ and 7 p.p.m. The 7-*cis* isomers were purified and isolated by HPLC on Corasil II or μ -Porasil columns. The major differences between the 7-*trans* and 7-*cis* isomers are the shift of H_{18} and H_7 from about $\delta 1.7$ p.p.m. resp. 6.3 p.p.m. (7-*trans*) to about $\delta 1.5$ p.p.m. resp. 6.0 p.p.m. (7-*cis*) and the decrease of the coupling constant $J_{7,8}$ from 16 Hz (7-*trans*) to about 12 Hz (7-*cis*).

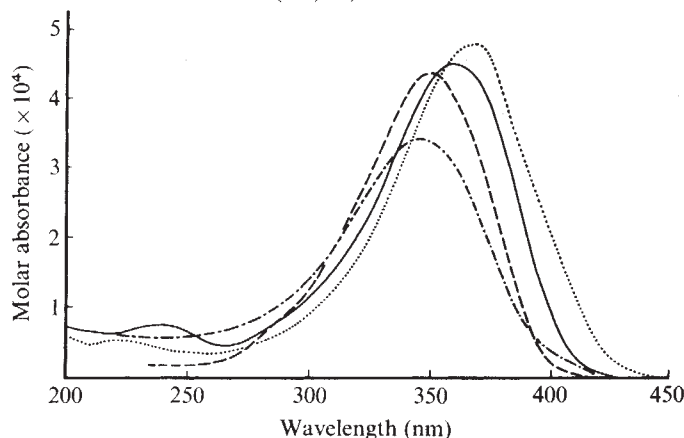


sion or in digitonin solution, the 7-*cis*-retinals as well as the all-*trans* slowly gave new absorption bands near 450 nm. The all-*trans* species, however, is due to random protonated Schiff bases between retinal and the various amoni groups present. With 0.1 M-hydroxylamine these are converted within seconds into retinal oxime (λ_{max} 367 nm), with NaBH_4 to retinylamines (λ_{max} 325 nm)⁸, while addition of 11-*cis*-retinal immediately generates rhodopsin. On the other hand, the new species formed by all the 7-*cis* isomers are stable in darkness to these reagents. Illumination destroys the 7-*cis* pigments, forming all-*trans*-retinal and opsin. The opsin can again recombine with 11-*cis*-retinal forming rhodopsin.

These data exclude the possibility that the new pigments are due to nonspecific interactions with opsin. The difference spectra before and after illumination in the presence of hydroxylamine show maxima at 450 ± 2 nm (7-*cis*), 460 ± 2 nm (7,9-*dici*s) and 455 ± 3 nm (7,9,13-*trici*s) (Fig. 3). The rate of pigment formation with the 7-*cis* retinals is only about 1/150 as rapid as with 11-*cis*-retinal.

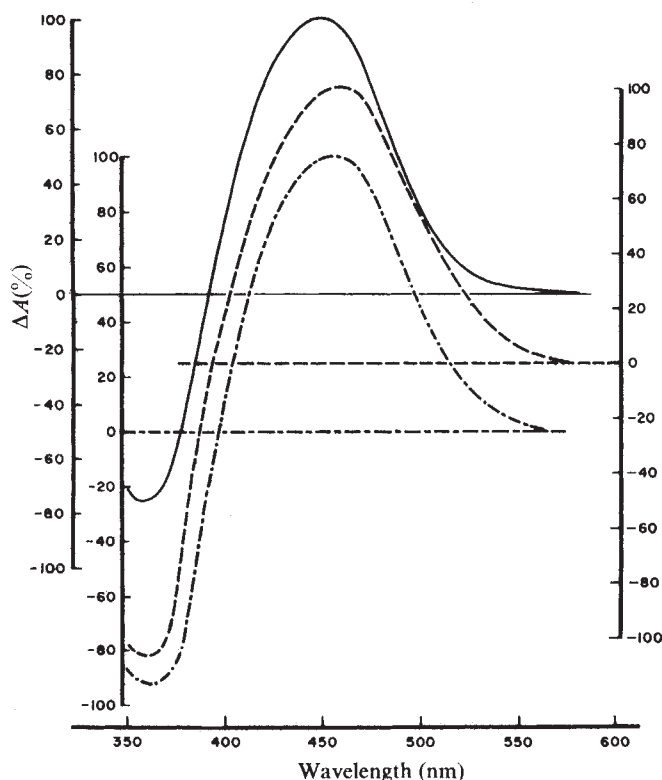
The 7-*cis* photopigments are clearly less stable than rhodopsin. They are bleached in the dark in 1% Triton X-100 or CTAB at room temperature, and seem to have a narrower pH-stability profile than rhodopsin. Attempts to purify the new pigments chromatographically have been hampered by this low thermal stability. Preliminary results indicate, however, that the difference spectrum maxima correspond with true absorption maxima. Furthermore, the molar absorbances are not far different from that of rhodopsin.

Fig. 2 Absorbance spectra of all-*trans*-(...), 7-*cis*-(—), 7,9-*dici*s-(---) and 7,9,13-*trici*s-retinal (----) in *n*-heptane. λ_{max} , in parentheses, and molar absorbance coefficients (last two from ref. 6): all-*trans*, (368) 48,000; 7-*cis*, (359) 44,100; 7,9-*dici*s, (351) 42,500; 7,9,13-*trici*s, (346) 36,600; 11-*cis*, (365) 26,400; 9-*cis*, (363) 37,700.



Our observations indicate that the active site of opsin is much less exclusive than originally thought. It seems to accept most of the known geometrical isomers of retinal (*cis* at 7-, 9-, 11-, 7,9-, 7,9,13- and 9,13 (ref. 11). Only 13-*cis*- and all-*trans*-retinals do

Fig. 3 Difference spectra of digitonin solutions of the pigments formed from opsin and various 7-*cis* isomers, obtained by subtracting the spectra of the bleached from those of the unbleached pigments in the presence of 0.1 M hydroxylamine. Illumination by a GE 150-W reflector flood light bulb at 35 cm through a 3-cm 6% CuSO_4 solution as a heat filter and a Balzers 482-nm interference filter. A mixture of 7,9,13-*trici*s- and 7,13-*dici*s-retinal (about 1:3) also gives a pigment with a λ_{max} at about 457 nm, indicating that 7,13-*dici*s-retinal also reacts with opsin. Although the properties of the new pigments clearly indicate that they are different from each other and from known pigments (rhodopsin, isorhodopsin I, II), we have not yet established rigorously the geometry of the retinals in the new pigments. Indirect evidence is our observation that the λ_{max} of the oximes liberated during thermal denaturation in 1% CTAB+0.1 M hydroxylamine corresponds with that obtained with the original retinal isomer (λ_{max} oximes: 7-*cis*, 353 nm; 7,9-*dici*s, 345 nm; 7,9,13-*trici*s, 344 nm; all-*trans*, 359 nm). —, 7-*cis*; ---, 7,9-*dici*s; - - - - - , 7,9,13-*trici*s.



not give rise to special pigments. But the rate of pigment formation and λ_{max} of the resulting pigments differ considerably for the various accepted isomers, 11-*cis* showing by far the highest rate and the largest redshift. As for the 7-*cis* analogues, the low reaction rate, low thermal stability and the smallest redshift observed suggest that conformational changes in the protein and/or the retinals take place in the formation of 7-*cis* pigments and that the final fit involves less interaction between the sidechain of the 7-*cis*-retinals and opsin than in the case of 11-*cis*-retinal. Apparently in the 7-*cis* analogues the protein is in a thermodynamically less stable state and contributes less to the absorbance characteristics of the pigment. The 7,8-*cis* bond introduces a large twist between ring and sidechain and therefore affects the shape of the retinal molecule to a much greater extent than the 9-, 11- or 13-*cis* bonds. This might explain why in the case of 7-*cis* isomers the geometry of the rest of the chain appears to be much less important than in the case of 7-*trans* isomers and has little influence on the reactivity with opsin. As compared with protonated Schiff bases of 7-*cis*-retinals with *n*-butylamine (λ_{max} 410–425 nm in Triton X-100 solution) the 7-*cis* pigments show a smaller redshift (20–25 nm for 7-*cis* and 35–40 nm for the 7,9-*dicis* and 7,9,13-*tricis*) than those in isorhodopsin and rhodopsin (50–60 nm). Similar to 11-*cis*, we suggest the shifts of the 7-*cis* isomers result from some secondary interaction (but to a lesser extent) between opsin and retinal, which is enhanced by the 9-*cis* geometry.

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Oxygen and toxicity inhibition of amino acid biosynthesis

ALTHOUGH oxygen is of therapeutic benefit for various human diseases, its usefulness is limited by its toxicity^{1,2}. In spite of progress, the cause of the convulsions observed in humans and other animals exposed to hyperbaric oxygen remains unknown^{3,4}, and the biochemical basis of the lung damage resulting from chronic exposure to increased oxygen tensions is poorly understood⁵. Oxygen toxicity, although necessarily expressed in different ways, affects various life forms from bacteria to man^{3,4}. There is considerable theoretical basis and experimental evidence for mechanisms of toxicity shared at the

cellular and subcellular levels, and research on basic sites of toxicity has often involved tissue cultures or bacteria². The growth and respiration of *Escherichia coli* is rapidly, but reversibly, inhibited by hyperbaric oxygen in minimal salts medium⁶. Significant protection for some microbes against the growth-inhibitory effects of hyperbaric oxygen has been obtained with nutritional supplements, including yeast extract⁷. We have now found that *E. coli* E-26, the source of which has been identified previously⁸, is protected from oxygen toxicity by yeast extract because of its amino acid content: a mixture of 20 amino acids gave similar protection.

Table 1 Contributions of individual amino acids to protection from oxygen-induced growth inhibition

Amino acid deleted	Generation time in a gas phase of		Normalised* ratio
	1 atm air	4.2 atm O ₂	
None	27.6 ± 1.2	49.5 ± 0.9	1.00 ± 0.05
All	41.5 ± 1.1	978 ± 102	12.98 ± 1.27†
Alanine	29.8 ± 2.1	52.7 ± 1.3	0.99 ± 0.07
Arginine	31.4 ± 2.0	48.3 ± 1.2	0.87 ± 0.05
Asparagine	29.2 ± 1.4	75.4 ± 4.5	1.45 ± 0.12†
Aspartic acid	29.6 ± 2.0	49.5 ± 0.7	0.95 ± 0.06
Cysteine	30.1 ± 1.4	92.5 ± 8.7	1.77 ± 0.24†
Glutamine	32.7 ± 1.6	54.9 ± 1.2	0.94 ± 0.06
Glutamic acid	27.9 ± 1.0	51.0 ± 0.8	1.02 ± 0.04
Glycine	26.6 ± 1.4	51.1 ± 1.7	1.09 ± 0.09
Histidine	35.4 ± 1.8	55.9 ± 2.3	0.89 ± 0.07
Isoleucine	32.4 ± 1.1	243 ± 50	3.39 ± 0.60†
Leucine	31.2 ± 1.3	144 ± 8.7	2.58 ± 0.22†
Lysine	30.2 ± 0.9	67.2 ± 5.9	1.26 ± 0.14
Methionine	31.9 ± 0.8	88.4 ± 8.4	1.55 ± 0.17†
Phenylalanine	30.7 ± 1.0	93.8 ± 3.6	1.70 ± 0.10†
Proline	28.3 ± 0.8	52.5 ± 1.9	1.03 ± 0.05
Serine	34.5 ± 2.0	52.5 ± 2.5	0.84 ± 0.02
Threonine	30.1 ± 1.4	71.4 ± 5.1	1.36 ± 0.13†
Tryptophane	29.5 ± 1.3	178 ± 14	3.33 ± 0.23†
Tyrosine	31.1 ± 1.9	287 ± 63	5.40 ± 1.29†
Valine	35.6 ± 1.7	901 ± 181	13.95 ± 2.80†
10 Non-protective‡ amino acids	29.0 ± 0.9	72.5 ± 1.8	1.38 ± 0.03†
10 Non-protective§ amino acids plus threonine	40.1 ± 3.9	106 ± 2.5	1.50 ± 0.11†
None; vitamins added¶	31.0 ± 0.9	58.2 ± 0.8	1.04 ± 0.03

A defined basal salts medium containing glucose as the sole carbon source⁶ was supplemented with mixtures of the 20 amino acids listed (each 0.65 mM) minus the indicated amino acids. Cultures were adapted to growth in the individual test media with air as the gas phase and grown in small vials with stirring in a vessel which could be pressurised. The vessel was incubated in a constant temperature bath at 37 °C. During exponential growth at an absorbance of 0.1 to 0.2 at 500 nm, the cultures were subjected to a gas phase of 1 atm of air plus 4 atm of oxygen by pressurisation with pure oxygen. After 1 h, the vessel was decompressed. All samples for absorbance measurements were collected in tubes chilled in ice and measured immediately with a Gilford spectrophotometer (the relationship between absorbance and plate colony counts was linear up to 0.8 A; all measurements were made below 0.8). Generation times with a gas phase of 1 atm of air were calculated from the slope of best fit relating four determinations of the logarithm of absorbance at 500 nm to minutes of incubation; generation times in hyperbaric oxygen were extrapolated from changes in the logarithm of absorbance during 1 h in hyperbaric oxygen. Values are averages ± s.e. with $n = 8$, except $n = 12$ for methionine and threonine; $n = 4$ for omission of all 20 amino acids; $n = 4$ for omission of 10 amino acids plus threonine, and $n = 4$ for 20 amino acids plus vitamins.

*The quotient obtained by dividing the ratio of the hyperbaric oxygen to air generation times by the value of this ratio with none of the amino acids omitted. This ratio compensates for the differences in growth rates in the various media and is normalised with respect to the protection with all amino acids included. The values of normalised ratios increase as protection of growth in hyperbaric oxygen is reduced.

†Significantly different at $P < 0.05$ from the value obtained with none of the amino acids omitted using Student's t test.

‡The amino acids included were: asparagine, cysteine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine.

§The amino acids included were the same as indicated in the previous footnote, except that threonine was not included.

¶p-Aminobenzoic acid, niacin, and pantothenic acid, each 0.65 mM.

Single deletions of amino acids from the mixture (Table 1) revealed that 10 amino acids were not required for protection against growth inhibition in hyperbaric oxygen, but that deletion of valine, tyrosine, isoleucine, tryptophan, leucine, phenylalanine, cysteine, methionine, asparagine or threonine (listed in order of decreasing effect) caused significant ($P \leq 0.05$) loss of the protection afforded by the mixture of 20 amino acids.

The simplest interpretation of these data is that hyperbaric oxygen significantly reduced synthesis of certain amino acids (those which provided protection when included). Presumably, oxygen inhibits specific enzymes involved in synthesis of these amino acids, although proof awaits direct enzyme assay.

To reduce the number of enzymes that might be involved, intermediates of biosynthetic pathways for several amino acids were substituted for the appropriate amino acids to test their ability to protect cells from hyperbaric oxygen (Table 2). α -Ketoisovalerate substituted for leucine (Table 2), suggesting that the pathway, containing four enzymes, from α -ketoisovalerate to leucine, is functional in oxygen-poisoned cells. In the parallel pathway for synthesis of leucine, valine and isoleucine, there is apparently a block (or blocks) in oxygen-poisoned cells of acetohydroxy acid synthetase, acetoxo acid reductoisomerase or dihydroxy acid dehydratase, for α -ketobutyrate did not substitute for isoleucine. Valine transaminase was not sensitive because α -ketoisovalerate substituted for valine. Leucine seemed to be the least critical of the branched-chain amino acids, perhaps because valine transamination is reversible and valine in the medium could provide a source of α -ketoisovalerate for the non-blocked series of reactions leading to leucine synthesis. Valine was among the most critical of the amino acids for protection from oxygen toxicity (Table 1). With valine biosynthesis blocked before α -ketoisovalerate, and no valine in the medium, the cells not only would lack valine for protein synthesis, but could make neither pantothenate nor products (such as coenzyme A) from pantothenate.

The synthesis of cysteine from serine was apparently impaired in hyperbaric oxygen (Table 1). Homocysteine substituted for methionine (Table 2), indicating that inhibition occurred before homocysteine, and that the methyl transferase was relatively insensitive to hyperbaric oxygen. The sensitive site in methionine synthesis probably also accounts for the requirement for threonine during exposure to hyperbaric oxygen (Table 1). Asparagine was apparently required during exposure to hyperbaric oxygen because of inhibition of asparagine synthetase.

The vitamins and vitamin precursor tested (Table 2) revealed

no additional protection even though their synthesis was impaired in hyperbaric oxygen (Tables 1 and 2). But failure to synthesise these vitamins probably would not have become growth limiting during the 1 h of exposure.

Explanation of the results obtained with precursors of aromatic amino acids requires consideration of the presence of three isozymes for the production of deoxyheptulosonic acid 2-phosphate and two isozymes for production of prephenic acid, and separate feedback and repression by phenylalanine, tyrosine and tryptophan of the isozymes and the enzyme involved in conversion of chorismate to anthranilate. The permeability of the cell to the intermediates is also a factor. To minimise these problems, cells were preadapted to each different medium before exposure to hyperbaric oxygen.

Shikimate did not substitute for all three aromatic amino acids when absent simultaneously during hyperbaric intoxication, whereas chorismate partially substituted for them (Table 2). This indicated that hyperbaric oxygen significantly impaired the conversion of shikimate to chorismate, but that there was also impairment before chorismate. Deletions of individual aromatic amino acids, with inclusions of each of four intermediates (Table 2), suggested several conclusions. Deletion of phenylalanine decreased protection less than deletion of either tryptophan or tyrosine (Table 2), suggesting that the enzymes in the part of the pathway common to the biosynthesis of all three aromatic amino acids (shikimate kinase, pyruvylshikimate-phosphate synthase or chorismate synthase) were less sensitive than one or more enzymes in the branch pathways from chorismate to tryptophan and tyrosine. There was no significant inhibition of the series of enzymes required for converting anthranilate to tryptophan.

The inconsistency in the failure of prephenate to substitute for phenylalanine (Table 2) could be explained by the known lack of permeability of cells to prephenate. But prephenate apparently penetrated these cells since it reduced the growth inhibition due to hyperbaric oxygen in tyrosine-deprived cells (Table 2). It is conceivable, however, that cells might adapt to prephenate transport when deprived of tyrosine, but not when deprived of phenylalanine.

The inclusion of only the 10 amino acids which, when individually omitted, caused a significant ($P \geq 0.05$) decrease in growth in hyperbaric oxygen (Table 1), gave almost as much protection as the 20 amino acids (normalised ratio of 1.38 ± 0.03 compared with 1.00 ± 0.05). The difference between these ratios, however, was significant ($P \geq 0.05$), indicating that other

Table 2 Inhibition of amino acid biosynthesis

Amino acid deleted	Precursor added	Generation time in a gas phase*		Normalised ratio
		1atm air	4.2 atm O ₂	
None	None	27.6 $\pm$ 1.2	49.5 $\pm$ 0.9	1.00 $\pm$ 0.05
Isoleucine	α -Ketobutyrate	32.2 $\pm$ 1.4	240 $\pm$ 12.6	4.14 $\pm$ 0.21†
Leucine	α -Ketoisovalerate	29.9 $\pm$ 0.4	64.4 $\pm$ 6.5	1.20 $\pm$ 0.12
Valine	α -Ketoisovalerate	27.3 $\pm$ 0.2	63.2 $\pm$ 2.0	1.28 $\pm$ 0.04
Methionine	Homocysteine	28.9 $\pm$ 0.6	60.7 $\pm$ 1.0	1.16 $\pm$ 0.17
Aromatics	Shikimate	32.5 $\pm$ 2.0	982 $\pm$ 325	17.32 $\pm$ 6.43†
Aromatics	Chorismate	34.8 $\pm$ 1.2	185 $\pm$ 40	2.99 $\pm$ 0.77†
Phenylalanine	Shikimate	29.6 $\pm$ 3.0	97.5 $\pm$ 6.1	1.87 $\pm$ 0.09†
Phenylalanine	Chorismate	42.5 $\pm$ 2.1	81.0 $\pm$ 1.2	1.06 $\pm$ 0.05
Phenylalanine	Prephenate	28.4 $\pm$ 0.6	92.5 $\pm$ 2.9	1.80 $\pm$ 0.08†
Phenylalanine	β -Phenylpyruvate	26.8 $\pm$ 0.7	63.8 $\pm$ 2.4	1.32 $\pm$ 0.08
Tyrosine	Shikimate	25.8 $\pm$ 0.2	295 $\pm$ 7.4	6.30 $\pm$ 0.13†
Tyrosine	Chorismate	30.4 $\pm$ 0.8	191 $\pm$ 19	3.53 $\pm$ 0.39†
Tyrosine	Prephenate	34.0 $\pm$ 2.0	157 $\pm$ 9.7	2.56 $\pm$ 0.19†
Tyrosine	<i>p</i> -Hydroxyphenylpyruvate	28.0 $\pm$ 0.6	69.6 $\pm$ 1.9	1.37 $\pm$ 0.04†
Tryptophan	Shikimate	30.6 $\pm$ 1.3	218 $\pm$ 12	3.94 $\pm$ 0.25†
Tryptophan	Chorismate	27.7 $\pm$ 0.6	186 $\pm$ 12	3.70 $\pm$ 0.26†
Tryptophan	Anthranilate	29.9 $\pm$ 1.2	72.8 $\pm$ 2.3	1.35 $\pm$ 0.06

Normalised ratio was obtained as described in Table 1.

Media and test conditions were as described for Table 1. Precursor concentrations were 0.65 mM except anthranilate (0.5 mM) and shikimate and chorismate (1.3 mM) when substituted for all three aromatic amino acids.

*As for Table 1, except $n = 4$ for all except $n = 8$ for shikimate with phenylalanine omitted, and $n = 11$ for chorismate with tyrosine omitted. Where more than one precursor for a given amino acid was tested, the precursors are listed in the table in the order of their biosynthesis.

†As for Table 1.

amino acids, which when omitted singly did not produce significant decrease in protection, may have small protective effects in concert.

The significance of the inhibition of amino acid biosynthesis (which also leads to inability to synthesise nicotinic acid, NAD, NADP, folic acid, pantothenic acid and coenzyme A) is immediately apparent with respect to the growth-inhibitory effects of hyperbaric oxygen for *E. coli* incubated in minimal salts medium, where all its amino acids and the indicated growth factors must be synthesised. The potential significance of these biosynthetic inhibitions for the functioning of mammalian systems is speculative. All the amino acids, except cysteine and asparagine whose biosyntheses were inhibited, are essential for man and rat. The inhibition of synthesis of essential amino acids is obviously irrelevant in higher mammals in terms of protein synthesis and growth. But because of the significant roles of amino acids as precursors for neurotransmitter synthesis, and as neurotransmitters in the central nervous system⁸, work is in progress to identify the specific oxygen-sensitive enzymes, and to investigate their potential roles in oxygen toxicity in higher systems.

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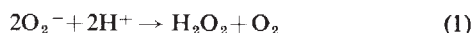
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Reactions involving singlet oxygen and the superoxide anion

In discussing reactions involving the superoxide anion O_2^- , it is generally considered that the radical itself or singlet oxygen generated from O_2^- is harmful to molecules of biological importance^{1,2}. Since the demonstration that singlet oxygen participates in reactions is technically difficult, conclusions about its effects are rather speculative. I show here, on thermodynamic grounds, in which reactions singlet oxygen can be formed from O_2^- and in which reactions this is improbable.

By way of illustration I shall use an oxidation state diagram for oxygen at pH 7, adapted from Phillips and Williams³ (see Fig. 1). In this diagram, the free energy per oxygen atom at pH 7 ($\Delta G_{O'}$) is plotted against the formal charge per oxygen atom (n). The ordinate at the left-hand side is related to the one at the right by $\Delta G_{O'} = nF\Delta E_{O'}$, in which F is the Faraday. By plotting the values this way, the slope of the line joining the species A and B represents the reduction potential $E_{O'}$ of the couple A/B. All of the species above the line joining O_2 to H_2O are thermodynamically unstable.

To explain the diagram, I have chosen as examples: the disproportionation of O_2 , the Haber–Weiss cycle, the reaction of O_2^- with superoxide dismutase and the reduction of ferricytochrome *c* by O_2^- . First the disproportionation of O_2 . O_2^- is not stable; it reacts with itself and forms oxygen and hydrogen peroxide



where $k = 4.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7, 22 °C (ref. 7).

Depending on the energy state of the oxygen molecule, the above reaction can have a $\Delta G_{O'}$ of –29.0 kcalorie ($^3\Sigma_g^-O_2$), –6.4 kcalorie ($^1\Delta_gO_2$) or +7.6 kcalorie ($^1\Sigma_g^+O_2$). Therefore, as the reaction involving $^1\Sigma_g^+O_2$ has a positive $\Delta G_{O'}$, O_2^- can be

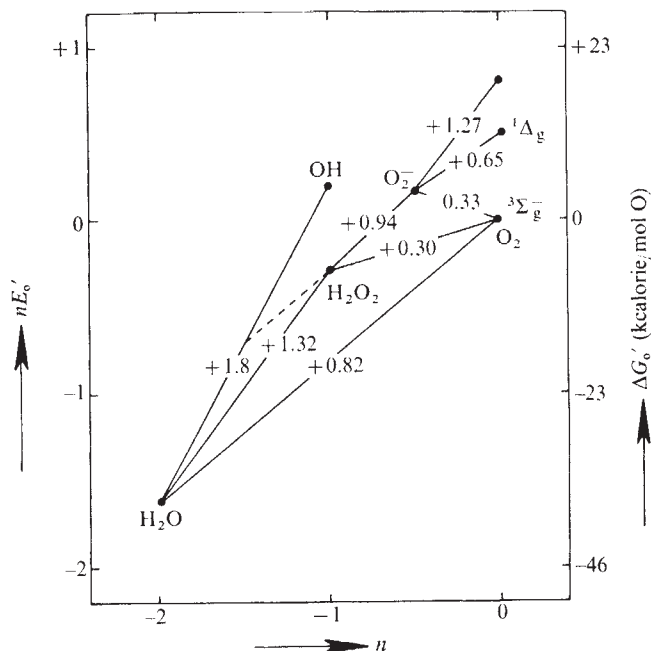


Fig. 1 Oxidation state diagram of oxygen ($P_{O_2} = 1 \text{ atm}$; $T = 25^\circ \text{C}$) at pH 7. Abscissa: formal charge, n , per O atom; ordinate, left: n times reduction potential $E_{O'}$; right: free energy per oxygen atom at pH 7, $\Delta G_{O'}$. The numbers refer to the slopes of the line. $E_{O'}$ (A/B) denotes the reduction potential of the couple A/B at pH 7 under otherwise standard conditions relative to the potential of the normal hydrogen electrode. For this diagram the following data were used: The triangle H_2O_2 , O_2 (triplet), O_2^- was constructed using the $E_{O'}$ values given by Muir Wood⁴. The differences in energy between the $^3\Sigma_g^-$ and $^1\Delta_g$, and between the $^3\Sigma_g^-$ and $^1\Sigma_g^+$ level of oxygen have been given as 22.6 and 37 kcalorie mol^{-1} , respectively⁵. The differences in entropy between the several forms of oxygen can be neglected with respect to the uncertainties in the values of the energy differences. The reduction potentials of the couples H_2O_2/H_2O , OH/H_2O and O_2/H_2O were computed from standard reduction potentials⁶.

a source of $^1\Delta_gO_2$ but not of $^1\Sigma_g^+O_2$.

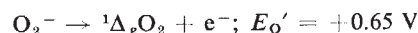
In 1934 Haber and Weiss⁸ proposed a mechanism for the disproportionation of hydrogen peroxide initiated by iron ions. The 'chain' reactions are



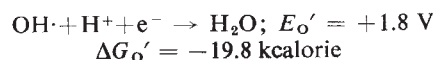
Kellog and Fridovich⁹ recently postulated that reaction (2) "liberates electronically excited singlet oxygen". Figure 1 shows that this is possible. The reaction can be split up



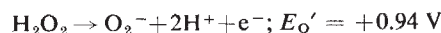
(see dotted line in figure) and



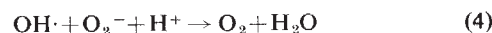
Since $E_{O'}$ (H_2O_2/OH , H_2O) is larger than $E_{O'}$ ($^1\Delta_gO_2/O_2^-$), the formation of $^1\Delta_gO_2$ is possible with $\Delta G_{O'} = -3.5$ kcalorie. Reaction (3) is also thermodynamically favourable



and



Arneson¹⁰ assumed that singlet oxygen was formed as a product of the chain-terminating reaction



It can be shown in the same way that the formation of triplet and both forms of singlet oxygen is possible.

The sum of reactions (2) and (3) is



From Fig. 1 it can be seen that hydrogen peroxide is unstable even with respect to $^1\Sigma_g^+\text{O}_2$ and H_2O : $\Delta G_o' = -5.0$ kcalorie per mol H_2O_2 . In the Haber-Weiss cycle, however, $^1\Sigma_g^+\text{O}_2$ cannot be formed. This may be because the energy released in reaction (3) is not available for the excitation of oxygen.

Superoxide dismutase (SOD) reacts with O_2^- at an almost diffusion-controlled rate ($k = (2.37 \pm 0.18) \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$)¹¹. The proposed mechanism is



The reduction potential of the couple $\text{SOD}-\text{Cu}^{2+}/\text{SOD}-\text{Cu}^+$ (+0.42 V (ref. 12)) is too low to oxidise O_2^- to $^1\Delta_g\text{O}_2$. The energy liberated by reaction (6) is 17.1 kcalorie and that from reaction (7) is 11.9 kcalorie. It has been argued¹³ that superoxide dismutase also catalyses the conversion of $^1\Delta_g\text{O}_2$ to $^3\Sigma_g^-\text{O}_2$: $\Delta G_o' = -22.6$ kcalorie per mol O_2 . It is not yet known whether this energy is dissipated over the protein molecule and finally transduced to water molecules, or whether it is used for a thermodynamically unfavourable reaction. Apparently the superoxide dismutase molecule can receive energies up to 22.6 kcalorie per mol SOD in single-step reactions without damaging itself. This is probably the reason why only one copper ion at a time is active, as the simultaneous reduction of both copper ions by O_2^- would liberate 34.2 kcalorie per mol SOD.

Under suitable conditions ferricytochrome *c* reacts¹⁴ with O_2^- (Koppenol and Van Buuren, unpublished). The couple ferri-/ferrocycytochrome *c* has a reduction potential of +0.26 V at pH 7 (ref. 15). This value is not high enough to cause $^1\Delta_g\text{O}_2$ to be formed. Measurements of the enzymatic activity after reaction with O_2^- indicate that the protein is still in its native form (Koppenol and Van Buuren, unpublished).

It is clear that when O_2^- reacts with a molecule it can act both as an oxidising or a reducing agent. It is possible that a protein can be damaged by such a reaction, but there is not much evidence for this in the literature¹.

It is unlikely that either of the forms of singlet oxygen will be produced if O_2^- reduces the protein molecule, as there are hardly any proteins with redox potentials as high as +0.65 V. Poupko and Rosenthal¹⁶ have reached the same conclusion, but their arguments have to be reconsidered, as they assumed that the ground state of O_2^- lies below that of oxygen. This assumption was based on a measurement in the gas phase¹⁷ and is not applicable to reactions in solution.

If O_2^- does not react with the protein it is possible that $^1\Delta_g\text{O}_2$ and H_2O_2 will be formed by reaction (1). This form of oxygen can react with various groups, for instance unsaturated bonds, and therefore has a destructive effect on the protein. It may be therefore that the main function of superoxide dismutase is to prevent the production of $^1\Delta_g\text{O}_2$ by reacting with O_2^- .

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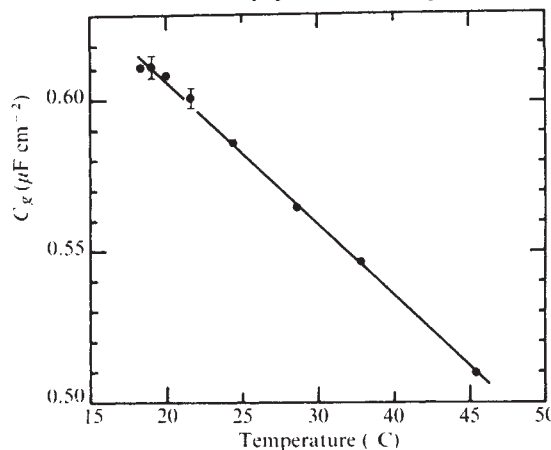
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The lipid bilayer as a 'solvent' for small hydrophobic molecules

THE cell membrane is viewed at present as a two-dimensional solution in which the lipid bilayer acts as a viscous 'solvent' for oriented integral proteins¹. Accepting this model as a working hypothesis, questions arise as to the nature of the hydrophobic interior of the bilayer and what rules proteins, lipoproteins, or polypeptides obey in interacting with it. The simplest hypothesis for the interior is that it is equivalent to a bulk alkyl solvent (albeit a very thin one). If this hypothesis is correct, the enthalpy of transfer (ΔH) of a solute molecule (for example, an alkane) from a bulk alkyl solvent to the bilayer interior should be small. That is, the solute-solvent interaction energy should be about the same in the bilayer as in the bulk alkyl. I have examined this hypothesis experimentally by measuring the solubility of *n*-hexadecane in planar bilayer membranes formed from glycerol-1-monooleate (1-GMO). The acyl chain of 1-GMO is approximately equivalent to 1-heptadecene. The enthalpy of transfer of *n*-hexadecane from pure liquid into 1-heptadecene can be estimated from solubility parameter theory² to be only a few calories per mol. Thus, the mixing of *n*-hexadecane in the bilayer interior should be nearly athermal: I have found, however, that ΔH is orders of magnitude larger than expected. This result has important implications for understanding the bilayer as a two-dimensional solution.

The thickness, and therefore the specific electrical capaci-

Fig. 1 Temperature dependence of the specific geometric capacitance (C_g) of planar bilayers formed from glycerol-1-monooleate (1-GMO). Aqueous phase: unbuffered 0.1 M NaCl. The curve through the points is a least squares fit to $C_g = a + bT$ where $a = 0.7005 \pm 0.0026$ (s.d.) $\mu\text{F cm}^{-2}$ and $b = -0.00471 \pm 0.00097$ (s.d.) $\mu\text{F cm}^{-2} \text{ } ^\circ\text{C}^{-1}$. Each point is the mean of measurements on three membranes (error bars or point diameter, s.e.m.). C_g of each membrane was determined in triplicate. Desorption measurements at the air-water interface using a Langmuir balance indicate that the 1-GMO is slightly soluble in the aqueous phase and that the solubility increases as temperature decreases (S. H. W. and N. L. Gershfeld, unpublished). The aqueous phase was therefore equilibrated at 20 $^\circ\text{C}$ with the bulk lipid solution (10 mg GMO per ml). Each membrane was allowed to 'age' for 10–20 min to ensure equilibration between microlenses and bilayer. In these conditions, C_g was invariant in time within the limit of $\pm 0.3\%$, and I believe the membranes were in physicochemical equilibrium.



tance, of planar bilayer membranes³ depends strongly on temperature^{4,5}, because the bilayer concentration of alkane solvent (used to form the bilayer) is temperature dependent. The bilayer is believed to be in physicochemical equilibrium with the bulk Plateau-Gibbs border (annulus) surrounding it and numerous microlenses^{6,7} of excess solvent trapped within it. Consequently, the planar bilayer may be viewed as a bilayer solution at saturation equilibrium with bulk alkane. Therefore, if the composition of the bilayer is measured as a function of temperature, the thermodynamics of transfer of alkane from the bulk state (microlenses and annulus) to the bilayer interior can be estimated using standard equations for saturated solutions². In this way, the interior of the bilayer can be compared directly with the interior of the bulk alkane solvent.

I estimated bilayer composition at various temperatures from measurements of specific geometric capacitance (C_g) using methods described in detail elsewhere^{5,8-10} (see Fig. 2 for summary), and the results at equilibrium conditions are shown in Fig. 1. The points can accurately be fitted with a straight line ($r = -0.9987$). The mole fraction (X_S) of *n*-hexadecane was calculated for each point and $\ln X_S$ plotted against $1/T$ and $\ln T$ (Fig. 2a and b). The enthalpy (ΔH) and entropy (ΔS) of transfer of hexadecane from bulk to bilayer were calculated from these plots and found to be $+3.79 \pm 0.10$ kcalorie mol⁻¹ and $+12.6 \pm 0.3$ entropy units (EU) respectively.

These large values of ΔH and ΔS indicate that the *n*-hexadecane molecules are not bound as tightly in the bilayer as in bulk and that they have more motional freedom. One explanation is that, in general, the cohesive forces in the bilayer are not as large as in bulk. The work of Gershfeld and Pagano¹² and Katz¹³ however, indicates that the cohesive forces between acyl chains in monolayers and bilayers are the same as in bulk. An alternative explanation is that the hexadecane molecules are located largely in the deep interior^{6,7} of the bilayer between the monolayers. Numerous X-ray diffraction studies of bilayers reveal a zone of low electron density in the bilayer mid-plane due to the methyl ($-\text{CH}_3$) groups (see, for example, ref. 18). There is thus little interdigitation between the apposed mono-

layers and a well defined interface must exist between the monolayers. Because the methyl groups have a lower polarisability than methylene ($-\text{CH}_2-$) groups, molecules located chiefly in the mid-plane should be subjected to smaller van der Waals' cohesive forces than if they are located parallel to methylene-rich acyl chains within the monolayers. Some hexadecanes must, of course be situated within the monolayers, but simple calculations show that these probably account for $\leq 20\%$ of the total

I therefore propose that the mid-plane of the bilayer is a special region because of the high concentration of methyl groups with smaller cohesive forces than found between the methylenes of the acyl chains. Support for this hypothesis is given by Dean and Hayes¹⁵ who measured the heat of sorption of hexane vapour on close-packed stearic acid monolayers at the vapour-water interface. At low partial pressures of hexane (that is, low surface coverage by adsorbed hexanes), the heat of sorption is 4.5 kcalorie mol⁻¹. At high partial pressures (high surface coverage approaching closest packing of hexanes on the stearic acid surface) the heat of sorption becomes identical to the heat of vaporisation of liquid hexane (7.5 kcalorie mol⁻¹). This indicates that the exposed $-\text{CH}_3$ surface of the stearic acid does not attract the hexanes as strongly as the fully covered hexane surface or bulk liquid.

Experiments are in progress to examine the thermodynamics of transfer of other alkanes into bilayers containing various acyl chains. Preliminary data for tetradecane-GMO bilayers give values of $\Delta H \approx 1.2$ kcalorie mol⁻¹ and $\Delta S \approx 4$ EU at 25 °C. The trend of the data suggests that shorter alkane molecules experience cohesive forces closer to bulk values. One reason for this is the larger volume fraction of the bilayer occupied by the smaller alkanes^{5,10} which means more nearest-neighbours will be alkanes rather than acyl methyl groups. Results¹⁴ on the solubility of hexane in various phospholipids indicates that significant differences can be expected among different types of acyl chains, and also that the interior of phospholipid bilayer dispersions is different from bulk alkyl solvents.

Finally, I should add that the results reported here for hexadecane depend on the assumptions made about how much the area per molecule of GMO varies with temperature. If the changes are large compared with those used to calculate X_S , then ΔH and ΔS could be much larger. Using a *de novo* method of calculating bilayer composition which predicts relatively large changes in area per molecule¹⁶, I have calculated $\Delta H \approx +9$ kcalorie mol⁻¹ and $\Delta S \approx 30$ EU. Thus, the values reported here probably represent lower limits.

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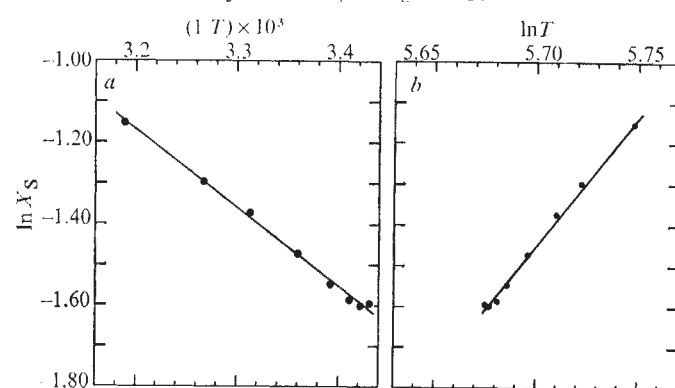
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Fig. 2 Temperature dependence of the composition of GMO-hexadecane membranes in a plot of *a*, $\ln X_S$ against $(1/T)$ and *b*, $\ln X_S$ against $\ln T$, where X_S is the mole fraction of *n*-hexadecane in the bilayer. X_S was estimated from the values of C_g using standard techniques^{5,10}. Briefly, bilayer thickness δ_B was calculated from $C_g = \epsilon_0 \epsilon_B / \delta_B$ where $\epsilon_0 = 8.85 \times 10^{-14}$ F cm⁻¹ and ϵ_B is the dielectric coefficient of the hydrophobic core. X_S can be calculated straightforwardly using the bulk densities and dielectric coefficients of the acyl chains (AC) (ref. 17) and hexadecane solvent (S), the number n_{AC} of acyl chains per unit area of bilayer, and the equations $\delta_B = v_{AC} n_{AC} + v_S n_S$, $X_S = n_S / (n_S + n_{AC})$; v represents molecular volume. Fettiplace *et al.*¹⁰ and Pagano *et al.*¹¹ have reported values for n_{AC} at single temperatures; I estimated values at other temperatures using correction procedures similar to those described elsewhere⁵. The corrections are slight and the errors incurred without them are small. At 20 °C, $n_{AC} = 5.26 \times 10^{14}$ cm⁻² (ref. 10) and $X_S = 0.205$. *a*, The curve is a linear regression ($r = -0.998$) whose slope ($-R[\partial \ln X_S / \partial (1/T)]$) gives the enthalpy (ΔH) of transfer of *n*-hexadecane from bulk to bilayer, $\Delta H = +3.79 \pm 0.10$ kcalorie mol⁻¹. *b*, Also a linear regression ($r = 0.998$) whose slope ($R[\partial \ln X_S / \partial \ln T]$) gives the entropy (ΔS) of transfer of *n*-hexadecane from bulk to bilayer, $\Delta S = +12.6 \pm 0.3$ EU.



matters arising

Quantification of secondary and tertiary structure base pairs in *E. coli* tRNA_{Val}

NUCLEAR magnetic resonance (NMR) is useful in the investigation of the base-pairing structure of tRNAs in solution because each Watson-Crick base pair and some tertiary structure base pairs give rise to resonances in the low field (11–15 p.p.m.) region of the spectrum^{1,2}. To determine the total number of base pairs present in tRNA it is necessary to integrate the low field NMR spectrum. We have not been satisfied with previous methods^{3–6} used to integrate tRNA spectra and present here a new method, based on using resonances in the aromatic region of the NMR spectrum. More accurate integration became necessary after X-ray diffraction results indicated that there are several tertiary structure base pairs^{7,8} in tRNA and after we found evidence for four common resonances in the low field region where are due to tertiary structure interactions².

In Fig. 1 the low field NMR spectrum of *Escherichia coli* tRNA_{Val} is compared with the resonances in the aromatic region of the spectrum in identical experimental conditions. The intensity in the aromatic region (8.5–6.5 p.p.m.) should correspond to 89 protons (one from each C, G, U and two from each A) and a comparison of the integrated intensity in the two regions indicates there are 23 ± 1 resonances (base pairs) in the low field spectrum. To check the method, we compared relative intensities in the aromatic (8.5–6.5 p.p.m.) and ribose regions (6.0–5.0) and found they agreed ($\pm 5\%$) with the value computed on the basis of sequence. Before commenting on these results, we will first discuss other methods which have been used and state why we believe that the use of the aromatic resonances is superior.

One method previously used by us and others to integrate tRNA spectra was to assume that one (or more) partially resolved resonance in the low field region of the spectrum has a known intensity, and then to use this resonance to calibrate the intensity of the rest of the spectrum^{3–6}. One problem is that it is not possible to prove that the reference resonance corres-

ponds to the assumed intensity. Another major problem arises from the Lorentzian line shape of the resonances. For example, for a resonance with a full half width ($\Delta\nu_{1/2}$) of 20 Hz, it is necessary to integrate over $6\Delta\nu_{1/2} = 120$ Hz to include 90% of its intensity. To include >95% of the intensity, the integration must be over $12\Delta\nu_{1/2}$, or 240 Hz. If neighbouring resonances are located within $2-4\Delta\nu_{1/2}$, there are additional problems with the allocation of intensity in the overlap region. Because of contributions from the Lorentzian tails, resolved resonances seem to have less intensity than do resonances which are bunched together. Therefore, if a resolved resonance is used as the intensity standard, the total intensity in the rest of the spectrum will seem to be larger by about 10%. When the collection of aromatic resonances are used as the intensity standard, however, the integration includes a relatively wide spectral range (10–20 $\Delta\nu_{1/2}$) and tails of various resonances add together to give a reliable value for the total intensity. It is possible that the line

shapes of the resonances observed in tRNA are not Lorentzian (as a result of incomplete motional narrowing, exchange or magnetic field inhomogeneity). A number of tRNA spectra have been simulated through the use of Lorentzian lines and, to within the limitations of experimental signal-to-noise ratio, the lines seem to be Lorentzian. Magnetic field inhomogeneity does not contribute to the line shape with the spectrophotometer we used.

Methyl resonances have also been used as internal standards to calibrate the intensity in the low field spectra³, but, in addition to the problems already mentioned, there are complications caused by impurities⁹ and undermethylation¹⁰. Impurities in the methyl region can be difficult to detect and the presence of a small amount of contamination can lead to larger percentage errors when this region is used as the integration standard rather than the aromatic region.

We have eliminated these problems by using the aromatic resonances as

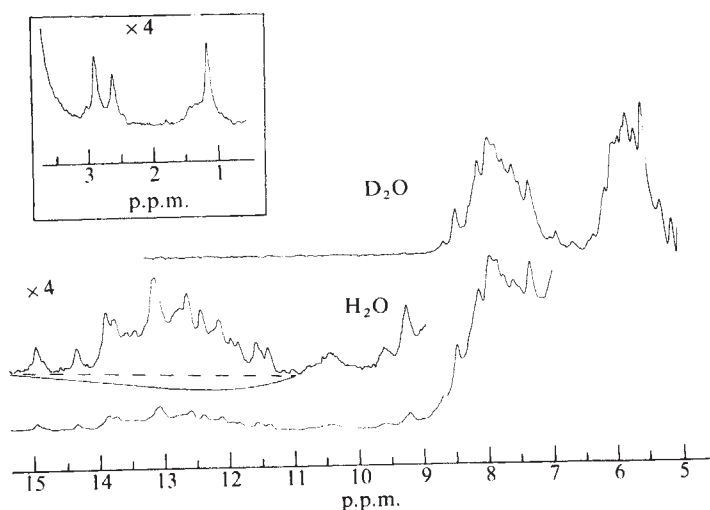


Fig. 1 300-MHz spectrum of tRNA_{Val} in various experimental conditions. The spectrum at the bottom is that of tRNA_{Val} in normal NMR conditions, that is, dialysed against 0.1 M NaCl, 10 mM MgCl₂ and 10 mM K₂H₂PO₄ pH 7.0. These spectrum of the methyl region was also obtained in those conditions. The sample was then dried to $\frac{1}{3}$ – $\frac{1}{4}$ of its original volume and then returned to its original volume with D₂O. This procedure was repeated several times until the sample was 95–98% D₂O. The spectrum of the aromatic region was obtained with the D₂O sample in experimental conditions identical to those for the H₂O sample. The methyl region of the spectrum was also investigated for the D₂O sample and the intensity compared with that of the H₂O sample. It was found to be within 5% indicating that there was no loss of the sample during the solvent change. The aromatic region contains 89 protons. Using the dashed baseline the low field region contains 23 protons, whereas use of the solid baseline gives an intensity of 26 protons for the low field region.

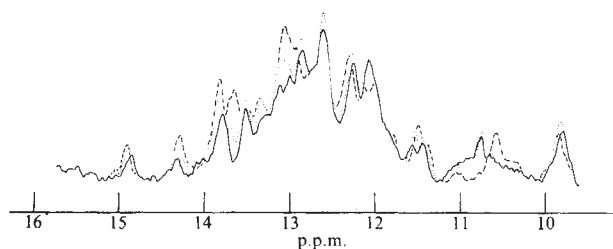


Fig. 2 300-MHz NMR spectrum of *E. coli* tRNA₁^{Val} in the presence of various amounts of magnesium at 35 °C. The sample was prepared by dissolving desalted tRNA in 0.1 M NaCl, 10 mM K₂H₂PO₄ pH 7.0. The intensities of the spectra correspond to 20 ± 1 (no Mg, —), 21.5 ± 1 (5 mM Mg, ···) and 23 ± 1 (15 mM Mg, ---) protons per molecule.

the intensity standard: the signal-to-noise ratio is excellent, there are no problems from impurities, and a large collection of resonances covering some 600 Hz are used to integrate a low field spectrum spanning 900 Hz.

The results with tRNA₁^{Val} corroborate our earlier suggestion that, in the presence of adequate levels of magnesium (or at sufficiently low temperatures), most *E. coli* tRNA contain 3–4 additional low field resonances due to tertiary structure base pairs². Our result for tRNA₁^{Val} (23 ± 1) is slightly lower than that (26 ± 1) recently obtained by Reid and Robillard³ using methyl and low field resonances as integration standards.

The results with *E. coli* tRNA₁^{Val} indicating the presence of 3–4 extra resonances in addition to those expected from the cloverleaf secondary structure, disagree with our earlier integrations on other tRNA which indicated that the total number of base pairs is somewhat lower (typically around 20)^{1,3,4}. The spectra in Fig. 2 illustrate the effect of magnesium on the low field spectrum of tRNA₁^{Val}. As the magnesium level is increased, the intensity in the low field region increases and most of this increase is at positions previously assigned to resonances from common tertiary interactions². By comparing spectra obtained with low and high levels of magnesium present, we estimate that half of the error in our previous integrations was a result of the low level of magnesium we used. The other half of the error can be attributed to the less accurate integration methods used.

Since this paper was submitted, we have re-examined a number of the tRNAs which we originally studied, and find that with higher magnesium levels and our new method of integration, most of the tRNAs contain 3–4 resonances in the low field region (11.5–15 p.p.m.) in addition to those expected from the cloverleaf secondary structure. Details of this work will be presented elsewhere.

The support of the US Public Health Service is gratefully acknowledged.

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¹ Kearns, D. R., and Shulman, R. G., *Accts Chem. Res.*, **7**, 33–39 (1974).

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⁸ Kim, S. H., et al., *Science*, **185**, 435–440 (1974).

⁹ Kastrup, R. V., and Schmidt, P. G., *Biochemistry*, **14**, 3612–3617 (1975).

¹⁰ Singhal, R. P., and Best, A. N., *Biochim. biophys. Acta*, **331**, 357–368 (1974).

REID REPLIES—Bolton and Kearns¹ take exception to our interpretation² of the NMR spectrum of tRNA₁^{Val}. Part of their problem arises from the poor resolution in their spectra which is due to either magnetic inhomogeneity or

sample impurity, or both. The bumps and shoulders in their spectrum are clearly recognisable as the individually resolved resonances in our spectra², but unfortunately cannot be integrated separately in the Bolton and Kearns spectrum¹. They also object to our use of several resolved single resonances as unit intensities, but we have already pointed out why these resonances cannot contain two protons². More serious objections can be raised concerning the Bolton and Kearns method of integration, which they presumably adopted as a result of their poor spectral resolution. First, the statement that the 8.5–6.5-p.p.m. region should contain 89 protons (the G aromatic proton, both A aromatic protons and one of the two aromatic protons of C and U) has not been verified experimentally and makes the dangerous assumption that no aromatic protons are shifted upfield or downfield out of this region by local ring current effects and that no ribose protons are shifted downfield into this region from the adjacent 'ribose region'. Second, their method of calculating the low field intensity suffers from the inaccuracies inherent in comparing two large numbers. In spite of these problems they have established an intensity that is within 4 protons of the correct value.

The fact that Kastrup and Schmidt's samples are contaminated with quaternary ammonium salts from RPC5 (ref. 3), and that careful chromatography resolves (and removes) undermethylated tRNA species⁴, is not relevant to either our spectra or our samples.

As Bolton and Kearns admit, the presence of several tertiary base pair resonances in tRNA spectra is in disagreement with their earlier integrations; it is also in disagreement with recently published integrations from that laboratory^{4–6} of spectra obtained in the presence of excess magnesium. Since these studies did not acknowledge the presence of tertiary resonances, one can seriously doubt the interpretations made from the observed changes in the NMR spectra.

Finally, I would also add that we are well aware of the Lorentzian nature and line shape of NMR resonances.

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¹ Bolton, P. H., and Kearns, D. R., *Nature*, **262**, 423–424 (1976).

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Matters arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words and the briefest of replies, to the effect that a point is taken, should be considered.

reviews

Waters of the Nile

Malcolm Coe

The Nile: Biology of an Ancient River. (Monographiae Biologicae, Vol. 29.) Edited by Julian Rzóška. Pp. 417. (Junk: The Hague, 1976.) Dutch Guilders 120.

No other river in the world can be as closely woven into the history of mankind as the Nile, sweeping for 32 degrees of latitude from the Equator to the Mediterranean. Such prehistoric and historic associations have been largely due to the fact that the "fluvatile" civilisations that have come and gone along its banks have been dependent not only on its life-giving waters but also on the huge and fertile silt burdens that it has carried from the interior of this vast continent.

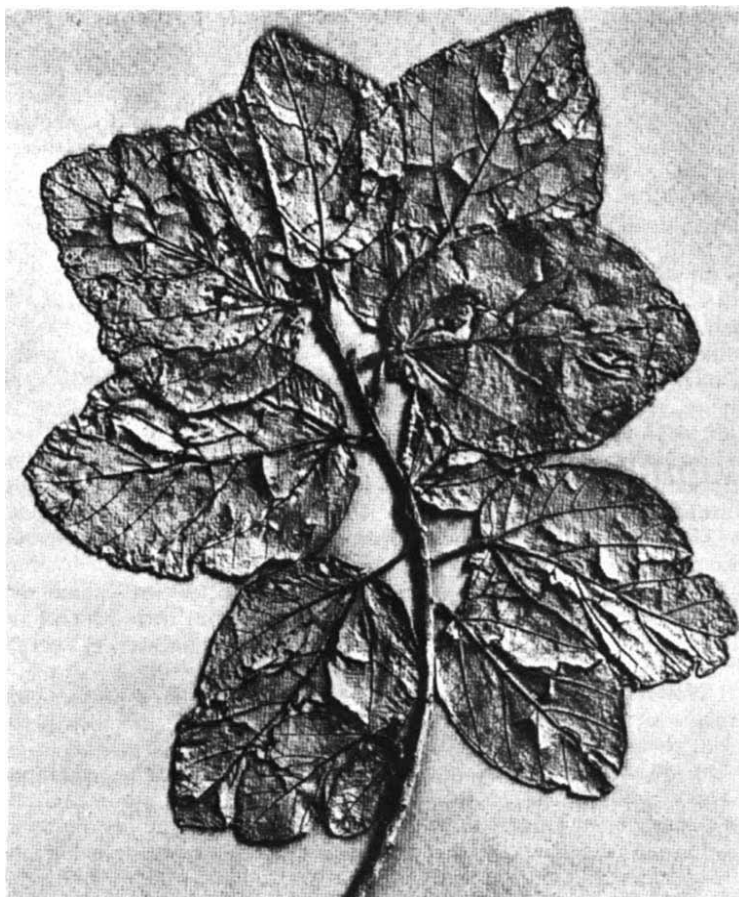
For us today the memory of Baker, Burton, and Speke who sought to find the source of the Nile are still comparatively fresh, yet the Nile's origins in snow-covered mountains (the Mountains of the Moon) are almost as old as human written history itself.

The history of the Nile valley begins with the regression of the Mediterranean in the Upper Miocene when about 5.5 Myr ago the sea dried up completely and as a consequence of this low sea bed level the resulting intense erosion led to the formation of the Nile valley. Later the great tectonic and climatic events of the Pleistocene resulted in great changes in the southern water sources, leading eventually to the formation of the Ne Nile about 30,000 yr ago. In spite of the long history of a 'Nile System', however, the river as we know it today is probably no older than 12,500 yr and possibly even as young as 10,000 yr.

Since this vast artery passes for about two-thirds of its length over arid (water-limited) country, the flora and fauna of much of its surroundings stands in stark contrast to the largely detritus-based biota of the river itself.

The fish fauna of the Nile has for so long formed an important item of diet for the civilisations that have waxed and waned along its banks. In all, the river and lakes located at its origins comprises some 320 species of fish of which 62% of them are endemic.

The problems related to development in the desert environment concern not only the problems of huge silt burdens but also concomitant biological effects such as the invasion of the water



Branch of sacred sycamore or mulberry fig (*Ficus sycomorus*) from a 20th dynasty Egyptian tomb. The tree is "not indigenous but (has been) cultivated in Egypt since times immemorial. It originates from Arabia and Somaliland. Its ancient name was Nehet-entep, and its fruits have been found in tombs of all ages, the earliest from the first dynasty. It was dedicated to Hathor, the Goddess of love and marriage. Even today a vestige of this belief can be seen in the custom of women visiting the sycamore when troubled by matrimonial cares".

hyacinth *Eichhornia crassipes* and the spread of the mollusc vectors of schistosomiasis.

Julian Rzóška has brought together a team of distinguished authors who have described the history, geomorphology, hydrology and biology of this great river and its environs in great detail.

The first four chapters deal largely with the history and prehistory of the Nile system, and later sections describe in detail the biotic and abiotic components of the system and its constituent parts.

Although most of this book is concerned with describing the main biotic and abiotic components of this important system, it is inevitable that a

vital part must deal with the effects of human interaction, particularly with regard to the Aswan High Dam.

Considering the huge problems that have faced the editor in attempting to condense such a vast assemblage of data this book will be a valuable source for all students of tropical limnology. If the presentation has a failing it is that the fragmentation under such a large number of chapter headings makes it difficult to view the Nile system as a whole rather than its constituent parts. □

Malcolm Coe is a member of the Animal Ecology Research Group in the Department of Zoology at the University of Oxford, UK.

Cost-benefit analysis for pesticides

Pesticides: Boon or Bane (Environmental Studies.) By M. B. Green. Pp. x+111. (Elek: London, May 1976.) £2.95.

THIS short book is intended primarily for use in university courses of environmental studies, and by those who are professionally involved with pesticides—farmers, industrialists and legislators. The author is involved in the development of new crop protection products by Imperial Chemical Industries.

The subject of gains and losses from the use of pesticides is developed round the theme of cost-benefit analysis, which is clearly distinguished from investment appraisal. Some of the difficulties are rather glossed-over. How does one optimise costs and benefits for the community as a whole? Those individuals and groups who bear the

external losses often gain no compensation except the altruistic satisfaction that their loss is somebody else's gain. Nor is a detailed example given, so that the subject lacks a feel of reality. Does, for example, control of red spider mite count as a credit or debit? Doubtless orchards sprayed to control red spider mite show a profit over those not so sprayed, but this mite only became a pest after insecticides were used.

The quantitative evidence presented for the agricultural benefits of pesticides is disappointing. Figures are given for both Great Britain and the USA of increases in crop yield, during recent decades, but this will depend on many things besides pesticides—for example, fertilisers, new plant varieties, and irrigation. Estimates are given, mostly for the USA, which has more severe pest problems than we do in Great Britain, for the financial gain from use of pesticides, albeit with no standard error or other indication of these estimates' reliability. The only example for

Great Britain (chapter 4) quotes work by Wheatley and Coaker to show that, for at least 30% of the Brussels sprouts grown in 1968/69, increased use of pesticides would have increased the financial loss.

Discussion of biological effects is very limited: virtually no discussion of pest resurgence, development of resistance to pesticides, or of the creation of new pests. The chapter on effects on wildlife is distinctly misleading.

Most of us are probably agreed that pesticides have an important role. This book shows how this role is seen by a scientist whose job it is to produce them.

F. Moriarty

Dr F. Moriarty has worked at Monks Wood Experimental Station since 1964. His research has been concerned principally with the biological effects of pollutants, especially organochlorine insecticides, on wildlife.

Developments in differentiation

Eukaryotes at the Subcellular Level: Development and Differentiation. (Methods in Molecular Biology.) Edited by Jerold Last. Pp. x+460. (Dekker: New York and Basel, January 1976.) \$32.50.

MESSENGER RNA (mRNA) metabolism is one of the important control points in the information flow from DNA to protein. In studies of development and differentiation therefore it is often desirable to measure the concentration or turnover of specific mRNAs. Until recently this could not be done because the concentrations were below the threshold of detection using the available methods. Two developments have altered the situation: first, the purification of specific eukaryotic mRNAs; second, the copying of mRNA by reverse transcriptase. As P. J. Williamson puts it in his chapter on nucleic acid hybridisation methods: "Mother Nature, working in collaboration with Temin and Baltimore, provided the key to an assay in an enzyme from RNA tumor viruses that copies RNA into DNA". By following the instructions in this manual, other research workers should likewise be able to make pure mRNA templates and complementary DNA, sensitive to as little as 1 pg of specific RNA or DNA, which is sufficient for many purposes.

This book strikes a good balance between highly specialised information, peculiar to the differentiating systems

at the focus of individual chapters, and more general methods. The latter include the isolation, separation characterisation and transcription of mRNA, and the use of nucleic hybridisation kinetics. The authors place due emphasis on various ways which have been found to avoid common pitfalls, for example, the synthesis of incomplete chains *in vitro*, polypeptides in the case of the popular wheat germ translation system (used to assay messenger activity), or DNA copies of mRNA in the case of reverse transcriptase. Mother Nature does not always collaborate with molecular biologists in her distribution of enzymes. Consequently, readers will appreciate the tips for eliminating or suppressing unwanted nucleases in various preparations. A high degree of specificity is required for many of the bioassays. Nonspecific precipitation can invalidate the results obtained using antibodies to characterise the products of *in vitro* translation, and several authors describe how they keep this to a minimum. A certain overlap between chapters concerning these tricky areas of experimentation is if anything helpful.

The mass of detail on gel electrophoresis of RNA and protein, repeated for a number of systems, is perhaps excessive, especially in view of the abundance of earlier reviews. On the other hand, there is too little space devoted to the manipulative aspects of hybridisation, particularly when the use of small (2 μ l) reaction mixtures in capillaries is recommended. The editor rightly celebrates the use of immunoprecipitation to isolate specific polysomes for purification of the less

abundant messengers. It was disappointing, however, not to find this method represented anywhere in the text, not even for the isolation of ovalbumin mRNA, which nicely demonstrates its potential. It was refreshing to read about biological systems as diverse as the insect chorion, slime moulds, red blood cells, lung, muscle and oviduct, in successive chapters. One important system is conspicuous by its absence; much progress has been made with immunoglobulin mRNA, and methods used in this work are sufficiently specialised to merit description.

The book is valuable as a timely source of information about new methods in molecular biology which are not collected together elsewhere. Like sucrose gradient centrifugation, the new methods will be of enduring value to future molecular biologists. The material in this book is well organised and thoroughly cross-referenced. My only complaint about this, the eighth in the series, is the grouping of the Figures at the ends of the chapters. To use this manual effectively, the reader must be dextrous in using his fingers to mark the appropriate parts of the text, the Figures, and the references, while turning the pages with his right hand. I am glad to possess this book: there should soon be many well-stained copies on laboratory benches everywhere.

H. J. Gould

Dr Gould is a lecturer in the Department of Biophysics at King's College, University of London, UK.

Scientific approach to art

Beyond Aesthetics: Investigations Into the Nature of Visual Art. Edited by Don Brothwell. Pp. 212+80 photographs. (Thames and Hudson: London, May 1976.) £9.50.

THE writers of the 12 essays collected here have tried to be objective about the visual arts. The task was fairly easy for two of them. Creedy describes the British system of art education, and says it is the best in the world. There is a light-hearted piece on the demography of artists by Cox. Statistics are hard to come by, and classification is capricious, but their pay, hours of work, and expectation of life seem about average. The apparent hazards of being an artist in France become comprehensible when one discovers that the category includes acrobats. Research has unexpected hazards. A chimpanzee bit her keeper for the first time when a drawing was incautiously interfered with. Whiten uses this as evidence for the intensity of the artistic impulse once it is aroused. It is odd that apes, unlike some birds, show little artistry when wild. This latency is perhaps similar to that shown by Eskimos. Decorating clothing and tools was traditional, but Brothwell says that the strikingly vigorous statuettes, now so much admired, were not made before European contact.

Defects in colour vision are as common among students at several art schools as in the general population. Pickford comments that nearly half of the group was unaware of the defect until tested in early adulthood. He comments also on the greater than average incidence of myopia and astigmatism among artists. He questions the idea that artistic differences between races have a physiological basis, although he comments on the curious absence of precise words for *blue* in many languages. It was this that led to the suggestion that colour perception varied. In a second article Pickford tries to make sense of aesthetic preferences in spite of their fluidity. Fluidity is the theme of Rookmaaker's article on art and morals. Using the depiction of nakedness as an example, he argues that anything can become acceptable if it is introduced in sufficiently small steps.

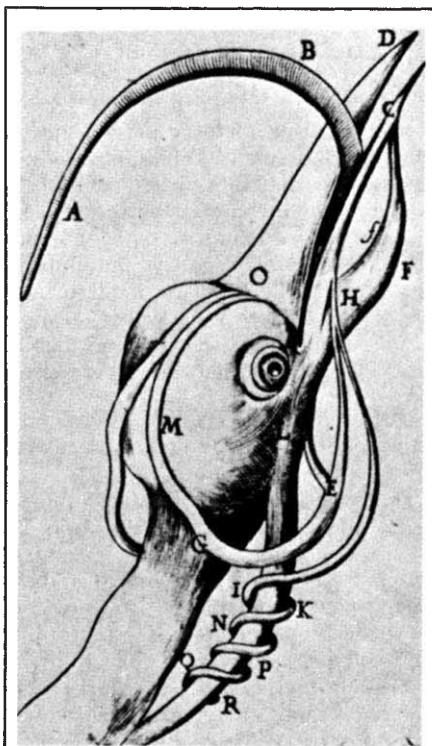
It is a pity Weale was not given more space to discuss the geometry and physics of art. Compression and too few illustrations make it difficult to understand all the interesting things he has to say on such subjects as deliberate distortion and the *pointilliste* technique. Writing of the use artists make of scientific illustrations he com-

ments "... if a pattern appears in a book it is part of science, but if it is brushed on to a canvas it becomes art."

There are two unsurprising articles on children's drawings and paintings, and an article on cross-cultural psychology that is surprising only because of the number of platitudes and tautologies crammed into it.

N. W. Pirie

N. W. Pirie has recently retired as head of the department of biochemistry at Rothamsted Experimental Station, Harpenden, UK.



Tongue and hyobranchial apparatus of a woodpecker. By G. A. Borelli, published in Rome in 1681. Taken from *A History of Comparative Anatomy: From Aristotle to the Eighteenth Century*. By F. J. Cole. Pp. 520. (Dover: New York; Constable: London, March 1976.) £3.90 paper.

Ion transport in plants

Uptake of Ions by Plant Roots. By D. J. F. Bowling. Pp. xi+212. (Chapman and Hall: London, Halsted: New York, March 1976.) £6.50.

THE uptake of ions by plant roots is a subject which over the years has probably had more man-hours devoted to it than any other single aspect of plant physiology. In consequence an extensive, very diverse and often contradictory literature has accumulated. From

time to time courageous individuals attempt to digest this material and produce a coherent exposition of the process. The would-be author is presumably initially inspired to his task by a conviction that he can see order where others cannot and inevitably therefore there will be; as Dr Bowling states in his preface, "some undue bias and personal foibles". His bias is towards the electrophysiology of roots, a topic which is well described. Realisation of this bias, however, seems to have caused the author to over-compensate to the extent that in a short book (185 pages of text) covering a vast subject he seems to have devoted a disproportionate amount of space to discussion of already well documented topics.

Although covering not merely the uptake of ions by roots, as the book's title implies, but also their transfer to shoots together with related information on leaves and algae has led to some superficiality in presentation, Dr Bowling has, however, produced an interesting and logically presented book. He looks on the overall process as being divisible into four sections or links in a chain: movement of salts in the soil to the root surface; uptake into the root; transport across the root and movement in the xylem to the shoot. Separate chapters deal with what Dr Bowling describes as "the more theoretical aspects of salt uptake".

Finally, there is a chapter which although headed 'Some conclusions and a look into the future' attempts firstly, to analyse results from his laboratory on the absorption of potassium and water by a sunflower plant grown in potting compost in controlled conditions in the light of the previous chapters; and secondly, describes briefly some recent experiments on the existence of specific ion binding proteins in the plasma membrane and the action of ionophores or ion-carrying agents. It is a pity that more space could not have been devoted to the relevance of these latter two aspects to the electrophysiological and kinetic approaches described earlier.

Because of its breadth of coverage the book is a good source of reference to the literature up to 1974 and it should prove useful to students and others specialising in the subject. I hope, however, that before the next printing is set, it will be re-proofed to remove some omissions in the references and misprints in the text.

D. A. Barber

Dr Barber is a Principal Scientific Officer at the Agricultural Research Council's Letcombe Laboratory, Wantage, UK.

Stellar photospheres

The Observation and Analysis of Stellar Photospheres. By David F. Gray. Pp. xv+471. (Wiley-Interscience: New York and London, January 1976.) \$27.70; £14.

THE study of stellar atmospheres (or even of just their photospheres, which are the layers emitting the bulk of the radiation) has become too vast a subject to be comprehensively covered in depth in a single book, and so it is natural (and indeed desirable) to find authors concentrating on their own particular fields of research interest while summarising other aspects more briefly. The distinctive and very valuable feature of the present book is that it provides a detailed elementary introduction to the experimental measurement and analysis of stellar spectral characteristics using predominantly Fourier techniques—a subject in which the author has himself done some very useful work and which is coming to be an essential tool in all spectral analysis with the advent of high speed multi-channel detectors and digital microdensitometers.

In other respects, the book is a clear,

well written and exceptionally well illustrated introduction from the practical point of view with enough of the theory of stellar atmospheres given (accompanied by many numerical formulae and tables) to show the application to the determination of effective temperature, pressure, gravity, radius, chemical composition and velocity fields. Difficult theoretical issues, like the intricacies of iteration to flux constancy and the effect of departures from local thermodynamic equilibrium, are mentioned and summarised as accurately as is possible within a few words supplemented by references, which is quite suitable for a text of finite size. It is always a weakness in this kind of treatment, however, that the reader does not quite get a physical feeling for the subject.

The presence of a few minor errors in places (for example, in the treatment of inward-directed radiation in the theory of radiative transfer) does not help and on these matters the reader will do well to consult both the old classical texts of Woolley and Stibbs, Unsöld and Aller and the modern one by Mihalas. There is, however, a very fine discussion (not otherwise available in textbooks or perhaps anywhere else)

of the use of line profiles to sort out rotation and different sorts of 'turbulence'—a field in which the author's Fourier methods offer an enticing vista of new research.

In the spirit of the generally practical approach of this book, there are extensive sections on spectrometers and detectors. These are generally very good, although it is a matter for some regret that the roles of grating blaze angle and camera speed in the efficiency of spectrographs have not been explicitly brought out. The treatment of instrumental profile and scattered light corrections is excellent.

Particularly in its treatment of the practical aspects of stellar spectrum analysis, this is a valuable book that is strongly recommended for all astronomical libraries and could be a worthwhile investment for research workers and graduate students; the price is not too unreasonable in present-day conditions.

Bernard Pagel

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Membrane research

Mammalian Cell Membranes. Vol. 1: General Concepts. Edited by G. A. Jamieson and D. N. Robinson. Pp. 276. (Butterworth: London and Boston, Massachusetts, 1976.) £18.

It has been said that a week is a long time in politics and things are becoming almost as fluid in membrane research. Since 1973, for instance, when much of the material in this book was prepared, the complete sequence of a transmembrane protein has been established by Marchesi and his colleagues. The molecular basis of ion conductance across membranes has become clearer at least conceptually in artist's impressions if not in hard fact, and the proteins involved are being characterised. Similarly the importance of microfilaments and microtubules in membrane function in non-muscle cells has been realised.

Some of the treatment understandably has a slightly old-fashioned look. For example, in Rose's short chapter on the anatomy of the mammalian cell discussion of the various forms of junctional complexes in cells must be inadequate in view of the considerable advances made on their structure and function. Two other examples concern surface labelling procedures particularly with lactoperoxidase-catalysed iodination and SDS-polyacrylamide gel electrophoresis. The description is very

scanty considering the recent extensive use of these techniques.

The book is the first in a five-volume series, and according to the Editors is an introduction to "the most essential aspects of physical and chemical studies which have contributed to present knowledge of membrane structure." This volume, however, is sharply divided on the basis of these criteria. On the one hand there are clear and straightforward descriptions, respectively, of the cultivation of cells by Robinson and a brief account by Glick of selected methods of plasma membrane preparation largely from cultured cells. Physical methods are covered by Levine and Chapman, and enzyme markers are summarised in a useful chapter by Hinton and Reid. The remainder of the book consists of four short speculative chapters on specialised, even esoteric, topics. Gingell argues for the electrostatic control of diffusion of proteins in membranes. The basic point is that proteins carry high negative charges (mainly due to sialic acid groups) that must be overcome by ligand binding before condensation can occur. Condensation of transmembrane proteins, equated by Gingell with intramembranous particles, is considered to be obligatory for formation of ion channels through the membrane. Well, maybe; but hardly the basic facts required for an introduction to membrane science. One difficulty too is the as yet inconclusive

evidence, except in erythrocytes, that intramembranous particles can be clustered by ligand interaction with surface membrane proteins.

The infant science of the mechanical properties of cellular membranes is interestingly reviewed by Canham, and Lenard and Landsberger discuss some models of membrane structure. The biogenesis of mammalian membranes is summarised by Malhotra. I enjoyed reading these four chapters and, for my money, they recommend the book for this year's bookshelf.

On the whole each chapter is well written and free of serious factual or typographical errors. The book should be useful to research workers and to final year undergraduates. The person who discovers on p32 that trypsin "catalyses the hydrolysis of peptide bonds between the carboxy group of arginine or lysine and the amino group of an adjacent amino acid" is likely to have difficulty in following many of the complex arguments that follow elsewhere in the book. The editors are to be congratulated on setting a good standard for the first volume and I look forward to the arrival of the remaining volumes.

R. Colin Hughes

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obituary

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Jacques Lucien Monod died at his family home in Cannes on May 31, 1976 at the age of 66. He was one of the foremost leaders of the revolution in molecular biology, helping to lay the framework of our understanding of gene action and protein synthesis. Monod was a scientist of the first rank, both in his experimental work and in his ideas. By his scientific research and by his *Essay on the Natural Philosophy of Modern Biology* (translated into English as *Chance and Necessity*) he has left an indelible mark on the intellectual history of the 20th century.

Monod was born in Paris in 1910. When he was seven his family moved to Cannes where he lived and studied till he was eighteen, so that he thought of himself more a man of the Midi than a Parisian. His father was a painter, an unusual vocation at that time for one of Huguenot stock, coming from a family which had produced many doctors, pastors, civil servants and academics. His mother was American, a native of Milwaukee, the daughter of a New England mother and a Scottish immigrant father. Early

influences were his father, who in addition to his artistic sensibilities had a strong interest in science and in Darwin in particular; also his Greek master, a scholar whom he admired for his civilised attitude to life. Scientifically, he acknowledged among his immediate elders the impact of George Teissier, André Lwoff, Boris Ephrussi and Louis Rapkine. Perhaps most important of all was his year's stay, in 1936, with Morgan's group at Caltech. Their full, free and critical discussions, together with the easy personal relationships between people of different ages, were a revelation to him and strongly influenced his style of organising research.

In his scientific career Monod, though never seriously impeded, did not get off to a flying start as many young men do nowadays. It was at the beginning of the war, after a famous reply to André Lwoff ("L'adaptation enzymatique? Connais pas!") that he first became aware of the nature of the phenomenon he was studying. Only after the war, when he had joined the Pasteur under Lwoff, did he fully re-

cognise the depth of the problem which was to become his life's work. By then he was 37. But once having recognised it, he pursued it with tenacity, versatility and imagination. His logic was relentless; his intuition variable but profound. No wonder that many of his younger colleagues came to feel that to work with him was a scientific education in itself.

The results are now in every textbook. There is no space here to follow the story in all its details, nor to show how the commonplaces of today grew out of the confusions of yesterday. The concentration on β -galactosidase; the arrival of Mel Cohn ("acquisition précieuse") who knew about proteins and also how to use the Tiselius apparatus; the proof that the appearance of enzyme activity represented *de novo* synthesis (a big surprise at the time); the skilful use of lactose analogues to separate the inductive from the substrate activity and the discovery of the permease and the transacetylase. Finally the grand collaboration with François Jacob (from the other end of the corridor) leading to their immensely skilful dissection of the inductive process by genetics; the realisation, from certain mutants, that the synthesis of all three proteins could be turned on and off together (the operon); the postulate that the *i* gene product was a protein—the inhibitor—and the famous PaJaMo experiment of Pardee, Jacob and Monod which suggested that the message was unstable. It is difficult now to recall that at that time the ribosomal RNA was assumed to be the message. The realisation that the ribosome was a reading head, which could read any message, came as a great flash of understanding. After that the road to the genetic code was wide open.

Meanwhile Monod, with Jacob and Changeux, had recognised that the properties required for the inhibitor were, at first sight, unusual for a protein. On reflection, however, they realised that such properties were in fact common—their existence had simply not been appreciated. Thus was born the theory of allosterie. Various earlier workers (including those studying haemoglobin) had glimpsed the idea but had not recognised its generality and its profound importance. For now one could see how any metabolic pathway could be linked, control-wise, to any other. Without this type of mechanism even the simplest organisms

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could not regulate themselves and higher organisms could not exist.

In 1969 Monod gave the Robbins Lectures at Pomona College in California. He used the occasion to develop and make precise his general ideas about biology, man and society. The lectures became a book, originally written in English, rewritten by him in French under the title *Le Hasard et la Nécessité*. Its publication made a strong impact and it became a best-seller. It aroused the almost united opposition of the French intellectual establishment which has always preferred Marx, Freud and Teilhard de Chardin to Darwin and Mendel. Written with force and clarity, in an unmistakable personal style, it presented a view of the universe that to many lay readers appeared strange, sombre, arid and austere. This is all the more surprising since the central vision of life that it projected is shared by the great majority of working scientists of any distinction. It would be difficult to find a better example to display the deep rift between science and the rest of our culture.

Monod was not aloof from public affairs. He was a persistent critic of the French University system and of the way in which French science was supported. Thus it was not entirely surprising that in 1971 he accepted the invitation to become Director-General of the Pasteur Institute. This decision came partly from his strong sense of duty; he was naturally reluctant to have to give up his research and his writing, but the Institute was in a bad way, having become desperately short of money. Monod threw himself wholeheartedly into his new job. He tried many things: appeals for funds, both public and private; the setting up of a production subsidiary with the aim to exploit useful ideas from the research side of the Institute; various economies and a limited reduction of staff. Finally

he toyed with a scheme, strongly opposed by many of his colleagues, to move the laboratory outside Paris. But the opposition to such a move was too strong and the sums of money required too great; only the government could supply them, a source he somewhat distrusted, as he feared for the Institute's independence. Looking back one can see that the Pasteur had to change and to change radically. It needed a scientist of Monod's stature to make the changes possible and reasonably palatable.

Science was the dominant activity in Monod's life but it was not the only one. During the war he worked for the French underground, receiving recognition for this perilous work from both the French and the American governments. He became a keen mountaineer only to give it up for sailing; the characteristic mixture of discipline and freedom appealed strongly to his temperament. He would sail his 37' boat single-handed or with the assistance of a mere amateur. Having a wide intellectual curiosity, he was remarkably well read, both in classical and modern authors—Camus was a personal friend. But his main passion outside science was music. He both played the cello and conducted. In his twenties he even wondered whether to give up science for music, and all through his life he tried to find time to make music with his friends.

Monod was a man of great personal charm. His English was perfect, though simpler than his French. Thanks to his good ear and his American mother, he spoke it without any trace of the heavy accent which most Frenchmen find difficult to discard. Good-looking, though small of stature, he commanded attention by his intelligence, his clarity, his incisiveness and by the obvious breadth and depth of his interests. Never lacking in courage, he combined a debonair manner and an impish sense

of humour with a deep moral commitment to any issue he regarded as fundamental. He had great warmth for his friends and treated his students with affection and candour, as if they were members of his family. To others he could be charming but somewhat more remote. Though his creative powers flowered most abundantly in his scientific work he combined within himself, in a natural harmony, the scientist, the philosopher, the man of action and the musician. He might well have made a world reputation by concentrating on any one of these roles. Such a range of gifts is rare. It is fortunate for us that he chose science, otherwise the development of molecular biology would have been very different.

The formal outlines of Monod's career will be recorded here only briefly. He obtained his first degree in 1931 and his doctorate in 1941, both from Paris. He joined the Pasteur Institute in 1945 as Chef de Laboratoire, becoming Chef de Service in 1953, head of the department of Cellular Biochemistry in 1954 and Director-General in 1971.

His stay at Caltech in 1936 was supported by the Rockefeller Foundation. While at the Pasteur he held a chair at the University of Paris from 1959 to 1967, followed by a chair in Molecular Biology at the Collège de France from 1967 to 1972. From 1962 onwards he was a non-resident fellow of the Salk Institute.

Monod received many honours, including Foreign Membership of the Royal Society and the US National Academy, among others, and several prizes, culminating in the award of the Nobel Prize for Physiology and Medicine in 1965 which he shared with Lwoff and Jacob.

His wife, the former Odette Bruhl, whom he married in 1938, died in 1972. They had two sons, twins, who survive him. Both are scientists. **F.H.C.C.**

announcements

Meetings

August 30–31, **L'Anthropologie et la Biologie des Populations Andines**, Toulouse, France (Prof Georges Lambert, Centre d'Hémotypologie du CNRS, Colloque d'Anthropologie et Biologie des Populations Andines, Hôpital de Purpan, Avenue de Grande-Bretagne, 31052 Toulouse Cedex, France).

September 2–3, **564th Meeting of the Biochemical Society**, Dublin (The

Meetings Officer, The Biochemical Society, 7 Warwick Court, Holborn, London WC1R 5DP).

September 6–8, **Biochemistry of Lipids**, Paris (J. Polonovski, Faculté de Médecine Saint-Antoine, 27, rue Chaligny, F 75571 Paris Cedex 12, France).

September 21–23, **Science and Technology of Water Soluble Polymers**, Cardiff (The Short Courses Secretary, Planning Section, UWIST, King Edward VII Avenue, Cardiff CF1 3NU, UK).

September 24–25, **Joint Radiological Meeting of the RCR and the BIR**, Leeds (The General Secretary, BIR, 32 Welbeck Street, London W1M 7PG).

October 7–8, **Meeting of the South-eastern Cancer Research Association on Membrane Receptors, Regulation of Gene Expression and Chemical Carcinogens**, Atlanta, Georgia (Dr Edward Bresnick, Chairman of the Dept of Cellular and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30902).